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Compared to other fields, developments and innovations in the fields of medical and health sciences are very fast. In this century, where the human population is rapidly increasing and technology is developing rapidly, health problems are constantly changing and new solutions are constantly being brought to these problems. With the Covid 19 epidemic, it has emerged that a health problem affects all humanity and all areas of life. For this reason, this conference focused on the changes and innovations in the field of Medical and Health Sciences.

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Investigation of Metabolites Isolated from *Sphingomonas egypticus* DM, A Rhizosphere of *Datura metel*

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Abstract: Using naturally occurring bioactive compounds has been crucial in progressing contemporary medical practices. The rhizosphere, which refers to the narrow region of soil surrounding plant roots, serves as a highly dynamic environment for soil bacteria owing to the substantial quantity of organic compounds released into the soil by plant roots. This paper outlines the fermentation and subsequent processing of rhizosphere *Sphingomonas egypticus* DM. The culture media of *Sphingomonas* was cultivated and subsequently subjected to propagation. The ethyl acetate extract was then obtained and subjected to fractionation and purification utilizing various chromatographic techniques. This process led to the isolation of a compound under investigation that exhibited a distinct spot on thin-layer chromatography (TLC) plates, with a calculated retention factor (R_f = 0.56) using a mobile phase composed of methylene chloride and methanol (9:1). Upon spraying with anisaldehyde/sulphuric reagent and subsequent heating for a brief period, the compound displayed a violet colour. This compound was investigated in vitro to assess its antimicrobial and minimum inhibitory concentration (MIC) capabilities. The results demonstrated a significant inhibitory effect against phytopathogenic fungi, specifically *Rhizoctonia solani* (21.3 mm) and *Alternaria alternate* (18.3 mm). Additionally, a moderate inhibitory effect was observed against *Pseudomonas aeruginosa* (12.7 mm), whereas a somewhat less inhibitory effect was shown against *Streptococcus mutans* (9.3 mm). The study also demonstrated that (MIC) against *Fusarium oxysporum* was seen at a concentration of 125 µg/mL compared to various conventional antibiotics. Finally, the strain underwent PCR screening to detect PKS and lipopeptide 4'-phosphopantetheinyl transferase *sfp* genes. The PCR amplification assay demonstrated the presence of genes encoding the KS domain and Surfactin. Furthermore, the sequences of *Sphingomonas egypticus* DM have been officially recorded in the NCBI GenBank database and can be accessed using the accession codes OR469907 and OR499756.

Keywords: *Sphingomonas*, *Datura metel*, Antimicrobial activity, Polyketide synthase, Lipopeptide gene

Introduction

Despite the prevalence of synthetic products, bioactive natural products continue to exert a considerable influence on modern medical practice. Antibiotics have been essential to the development of modern medicine, yet they are currently losing some of their effectiveness. As a result, finding new natural products is a direct consequence of the research into new biodiversity niches (Radić & Štrukelj, 2012). This is where bio products come in, such as bacteria, which have shown that they successfully prolong the amount of time that food may be stored while also conserving the environment and keeping the nutritional value of the food intact (Huang et al., 2003). Biological control agents are also important in drug discovery because of the variety of their structures and the range of their biological activities (Newman & Cragg, 2016; Pastor et al., 2012). *Datura metel* has a long history of use in traditional medicine to treat many diseases, including those related to the nervous and cardiovascular systems, fever, catarrh, pain, diarrhea, skin diseases, chronic bronchitis, asthma, digestive disorders, etc. It is rich in a wide range of essential phytochemicals, such as withanolides, daturaolone, datumetine, daturglycosides, ophiobolin A, baimantuoluoline A, and a lot of other similar compounds (Islam et al., 2023). In addition, the seeds of *Datura metel* plant contain various combinations, the concentration of which rises as the plant matures. These components include alkaloids, tannins, cardiac glycosides, flavonoids, carbohydrates, amino acids, and phenolic substances (Afsharypuor et al., 1995; Ratan et al., 2011).

The rhizosphere is home to a rich microbial community that substantially influences plant development (Philippot et al., 2013; Raaijmakers et al., 2009; Zhou et al., 2021). The antimicrobial activity of rhizobacterial communities is due to specific metabolites, such as phenazines, phenolics, and pyrrole-type compounds (Kennedy et al., 2015). *Sphingomonas* is a genus of rod-shaped bacteria well-known for its ability to digest environmental toxins and industrial pollutants and persist in hostile conditions (Fegatella & Cavicchioli, 2000; Leys et al., 2004; Seo et al., 2009; Zhang et al., 2020). *Sphingomonas* strains have significant quantities of dihydrosphingosine, long-chain sphingolipid, and polyamines (Takeuchi et al., 2001). Extensive research has been conducted on this genus, and 139 species have been identified and described (Busse et al., 2003; Chen et al., 2012; Feng et al., 2017; Shen et al., 2022; Takeuchi et al., 2001; Yabuuchi et al., 2002). The phyllosphere and the rhizosphere are just two plant-based environments that have yielded *Sphingomonas* (Chung et al., 2011; Rivas et al., 2004; Zhu et al., 2015).

The structural characteristics of natural products have shown that most are members of a significant chemical family. This family is known as polyketides, composed of compounds manufactured by multifunctional enzymes known as polyketide synthases (PKSs) (Hildebrand et al., 2004). In symbiotic microbes, the biosynthetic sources of natural products have been conclusively proven in various studies based on the genomic method. This comes from the rapid growth of molecular genetics connected to polyketides in microorganisms (Davidson et al., 2001; Piel et al., 2004; Schmidt et al., 2005). Identifying the authentic microbial origin responsible for the biosynthesis of the desired natural compound from the host organism is essential. Discodermolide is commonly biosynthesized using bacterial type I modular polyketide synthase (Staunton & Weissman, 2001). Antimicrobial substances known as lipopeptides are often generated by *Bacillus* and *Pseudomonas* species and have a cyclic structure with a low molecular weight (Peypoux et al., 1978; Xun-Chao et al., 2013). These primarily comprise a hydrophilic head consisting of seven to ten amino acids coupled to a hydrophobic fatty acid tail (Vanittanakom et al., 1986). Surfactins comprise a chain of seven α-amino acids connected to a distinct β-hydroxy fatty acid. Surfactins have a range of fatty-acid chain lengths from C13 to C16 (Lang, 2002). Surfactins have been shown to exhibit potent antibacterial activity (Gond et al., 2015). It is necessary to research microorganisms' host specificity to determine whether they create particular secondary metabolites with potential ecological or therapeutic significance. In addition, the efficient selection of a specific strain of a bioactive microorganism is based on the presence of a gene responsible for the biosynthesis of a secondary metabolite. Combining the screening for bioactivity with the screening for genes involved in the biosynthesis of secondary metabolites is possibly one appropriate technique (Zhu et al., 2009).

Sphingomonas egypticus DM, a rhizosphere strain, has been shown to secrete a promising secondary metabolite, and this has sparked a lot of interest in the possibility of exploring the biological consequences of this metabolite. Moreover, using PKS and lipopeptide *sfp* phylogenies, this study attempted to present molecular evidence of the host specificity of *Sphingomonas egypticus* DM.

Materials and Methods

Collection of Bacterial Strain

Sphingomonas egypticus strain DM (OQ269457), previously isolated from the rhizosphere of *Datura metel* by the current authors, was obtained from the laboratory collection of the current authors.

Large-Scale Fermentation and Working Up for Extraction of Secondary Metabolites

As a seed culture, the bacterial suspension containing the selected effective strain was inoculated into 100 mL of tryptone soy broth medium and cultured at 37°C for two days. For propagation, 5 mL of the seed culture was transferred in an aseptic manner to inoculate a total of ten 1 L Erlenmeyer flasks. The flasks were incubated at 37°C for 14 days while continuously shaken. Following incubation, bacterial cultures were sonicated for 30 minutes to rupture the cells. After harvesting, the broth was macerated in methanol (5 L). Following filtration and vacuum concentration, the methanol extract was prepared. The remaining residue was extracted using ethyl acetate until exhaustion after suspending it in water. The ethyl acetate extract was vaporized until completely dry and preserved for subsequent purification (Hamed et al., 2021).

Purification and Identification of the Isolated Secondary Metabolites

2.5 grams of ethyl acetate fraction was subjected to normal phase silica gel column chromatography (60 x 2 cm). A stepwise elution of the column with DCM–MeOH gradient [DCM, DCM: MeOH (95:5)] was monitored by analytical TLC and preparative TLC (0.5mm thick) performed on pre-coated silica gel plates (Merck, Germany). R_f values of the bioactive compounds and visualization of their chromatograms were carried out under UV light (254 and 365 nm) and further by spraying with anisaldehyde/sulfuric acid reagent and heating (Springer-Verlag, 1984). Furthermore, the use of Dragendorff reagent is employed for the purpose of detecting the presence of alkaloids (Linch et al., 1973).

Screening of the Antimicrobial Activities

Test Microorganisms

Gram-positive bacteria (*Staphylococcus aureus ATCC:13565* and *Streptococcus mutans ATCC:25175*) and Gram-negative bacteria (*Escherichia coli ATCC:10536, Pseudomonas aeruginosa ATCC:27853* and *Klebsiella pneumonia ATCC:10031*) were provided by Micro Analytical Center, Faculty of Science, Cairo University. Additionally, Fungal Phytopathogens (*Alternaria alternate SCUF0000310, Rhizoctonia solani SCUF0000317,* and *Fusarium oxysporum SCUF000091*) were received from the Agricultural Genetic Engineering Research Institute, Cairo University, Cairo.

Antimicrobial Activity

The antimicrobial activity of the purified secondary metabolite was investigated against various human and phytopathogenic microorganisms using the agar well diffusion method to observe the zones of inhibition in comparison with conventional antibiotics. Using Mueller–Hinton agar media, the chemical was evaluated for its potential to inhibit the growth of bacteria *in vitro*. In addition, the antifungal activity was assessed using a sabouraud dextrose agar medium. For Gram-positive and Gram-negative bacteria, the antibiotics ampicillin and gentamicin, respectively, were used as standard drugs. For fungal strains, nystatin was considered as a standard drug. As a solvent control (a negative control), DMSO was utilized. A sterilized cork borer was used to create wells in the solidified media with a diameter of 6 mm. After that, a total volume of 100 µL of the tested compound was added to each well. Regarding antibacterial activity, the plates were kept at 37°C for 24 hours, but for antifungal activity, they were kept at 28 °C for three to five days. This experiment was performed in triplicate, and the inhibition zones were determined on a millimeter scale (Scott, 1989).

Determination of MICs

A single colony was selected from each strain and placed into a tube containing 3-4 mL of sterile broth medium. The mixture was incubated at 37°C for 2-6 hours, and the turbidity of the suspension was then compared to a McFarland Standard 0.5. Once the turbidity was equal to or greater than the standard, the antimicrobial agent was dissolved in 1 mL of DMSO, and two-fold serial dilution was done using a broth medium. A predetermined quantity of the bacterial inoculum was introduced into each tube and allowed to incubate for 16-20 hours (or 24-48 hours in the case of fungal inoculum). The Minimum Inhibitory Concentration (MIC) represents the minimum concentration of antimicrobial agent that effectively curtails the visible growth of the tested isolate, ascertained through naked-eye observation (Chudáčková et al., 2010).

Extraction and Purification of DNA

To transfer the freshly enriched *Sphingomonas* culture, a micro-centrifuge tube with a 1.5 mL capacity was utilized, with one milliliter of the culture being transferred. The cell suspension was centrifuged for 10 minutes at 14,000 × g. The pellet was then resuspended in 300 µL of DNase-RNase-free distilled water and centrifuged once more for 5 minutes at 14,000 × g. After carefully discarding the supernatant, the pellet was resuspended in 200 µL of DNase-RNase-free distilled water. The suspension was then incubated for 15 minutes at 100°C and rapidly chilled on ice. Finally, it centrifuged for 5 minutes at 14,000 × g at 4°C, with 5 µL of the supernatant used as the PCR template DNA (De Medici et al., 2003).

PCR Amplification

The Taq polymerase relied on DNA samples as its template, and all PCRs were conducted using a T1 Thermal Cycler from Biometra, Germany. The resulting products underwent screening via 1% agarose gel electrophoresis. To further analyze *Sphingomonas egypticus* DM, which exhibited notable antimicrobial activity, we searched for the 4'-phosphopantetheinyl transferase *sfp* gene for surfactin lipopeptide. The lipopeptide gene was amplified through PCR, starting with an initial denaturation of 5 min at 95°C, followed by 30 cycles of denaturation (1 min at 94°C), annealing (1 min at 52°C), extension (1 min at 72°C), and a final extension at 72°C for 10 min. For detecting modular PKS genes, degenerate oligonucleotide primers were utilized. The reactions involved the following steps and cycles: 3 min at 94°C, 35 cycles at 94°C for 1 min, 59°C for 1 min, and 72°C for 1 min, followed by 7 min at 72°C (Gond et al., 2015; Zhu et al., 2009). The primers used for respective genes are listed in Table 1.

Table 1. PCR primers for amplification of lipopeptide *sfp* and PKS genes.

Target gene	Primers	Sequences (5' - 3')	Amplicon size
sfp gene	*sfp*-f	ATGAAGATTTACGGAATTTA	675
	sfp-r	TTATAAAAGCTCTTCGTACG	
KS domain	KSF	GCGATGGATCCNCAGCAGCG	680
	KSR	GTGCCGGTNCCGTGNGYYTC	

DNA Sequencing

The PCR product purification from 1% agarose gel was accomplished using a GeneDireX gel extraction kit (Taiwan). After that, the Macrogen facility in Korea performed sequencing using the *sfp*-f and KSF forward primers with the Big TriDye sequencing kit (ABI Applied Biosystems) (Zhu et al., 2009).

Sequence Analysis and GenBank Accession Numbers

In conjunction with the National Center for Biotechnology Information (NIH, MD, U.S.A.), the BLAST tool was utilized to establish the similarity percentage of the nucleotide sequences compared to other sequences. To create a phylogenetic tree, the nucleotide sequences were aligned with different sequences sourced from GenBank, utilizing Clustal X (Thompson et al., 1997). From there, MEGA version 3.1 was used to construct neighbor-joining phylogenies (Kumar et al., 2002). Lastly, the sequences presented in this study from *Sphingomonas egypticus* DM have been deposited in the NCBI GenBank database.

Statistical Analysis

One-way analysis of variance (ANOVA) is used to thoroughly analyze the antimicrobial study data. After that, a Duncan multiple comparisons test was carried out with the SPSS software version "22" for Windows.

Results and Discussion

Large-Scale Fermentation And Working Up

Large-scale fermentation of the bacterium followed by solvent extraction and purification using various chromatographic methods (see experimental section) led to the isolation of potent secondary metabolite. The obtained fractions from the column chromatography of the bacterial crude culture extract (2.5 gm) were monitored by TLC, and the common fractions were collected. In our previous work (submitted to BMC complementary medicine and therapies), two compounds were identified and tested for their biological activity. Here, we report the isolation of further compound eluted from the column using a mobile phase composed of methylene chloride and methanol (9:1). Testing of this compound on TLC using the same solvent polarity revealed its purity, and it acquired a violet colour on spraying with anisaldehyde/sulphuric acid reagent. The purity of the isolated compound was also verified by preparative thin layer chromatography (PTLC) to eliminate any other contaminations present (Figure 1). The chemical structure of this compound is under investigation. Simultaneously, we continue testing its biological activities.

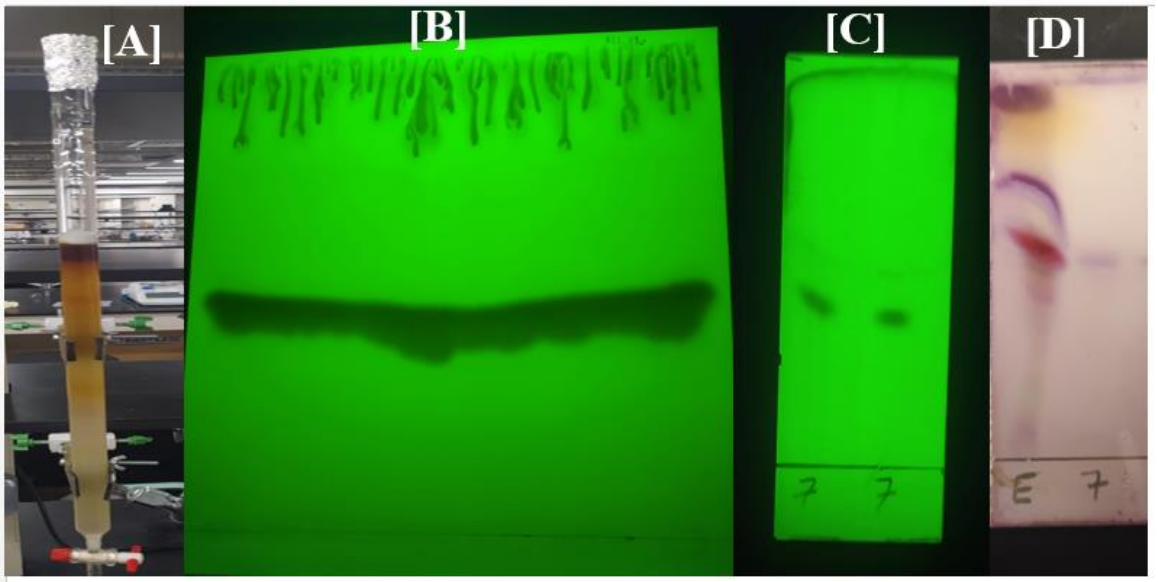

Figure 1. Various chromatographic methods used for isolation and purification of a potent secondary metabolite [A] Bacterial crude extract subjected to column chromatography, [B] Preparative TLC for purification, [C] TLC visualized by UV light (254 nm), [D] Spraying with anisaldehyde/sulphuric acid reagent. Dragendorff reagent gives negative result.

Biological Activities

Antimicrobial Screening

As part of the research, a refined secondary metabolite was derived from *Sphingomonas egypticus* DM and assessed for its ability to combat microbes. Following incubation, clear zones with measurements ranging from 9.3 to 21.3 mm were observed against various bacteria and fungi, as detailed in Table 2. These findings suggest that the isolated compound has the potential to serve as an antibacterial or antifungal agent against certain harmful microbes. Based on the agar well diffusion method, it was determined that the compound effectively inhibits at least three tested pathogens. It showed significant antimicrobial activity against pathogenic fungi, such as *Rhizoctonia solani* (21.3 mm), *Alternaria alternate* (18.3 mm), and *Fusarium oxysporum* (17.0 mm). Moreover, a moderate inhibitory effect has been shown against pathogenic bacteria such as *Pseudomonas aeruginosa* (12.7 mm) and *Klebsiella pneumonia* (12.3 mm). However, the metabolite exhibited the least inhibitory effect against *Streptococcus mutans* (9.3 mm), as illustrated in Figure 2.

Numerous types of bacteria, such as *Sphingomonas, Bacillus, Pseudomonas, Aeromonas, Marinobacter, Nocardia,* and *Methylobacterium,* have been researched for their ability to create antimicrobial substances that relate to the rhizosphere and phyllosphere (Bodenhausen et al., 2013; Buedenbender et al., 2017; Chen et al., 2019). Studies have indicated that *Sphingomonas* sp. is the primary organism in healthy dicotyledonous plants and can lessen disease symptoms while hindering the growth of the foliar plant pathogen *Pseudomonas syringae* (Innerebner et al., 2011). Studies indicate that *Sphingomonas* spp. has the potential to shield plants against Xcc, a notorious bacterium known for causing plant diseases (Buell, 2002). This suggests that they may be able to combat a broader spectrum of foliar pathogens. As Ji and Wilson explain, bacterial strains with a higher

nutritional resemblance to the pathogen are more likely to be effective against plant diseases (Ji & Wilson, 2002). Natural compounds, such as secondary metabolites synthesized by plants and microbes, have been extensively researched as a sustainable substitute for synthetic chemicals (Atanasov et al., 2015).

Table 2. Antimicrobial activities of pure secondary metabolite recovered from *Sphingomonas egypticus*.

Sample / Microorganism	Zone of inhibition (mm)	
	Pure Secondary metabolite	Standard antibiotic
Gram-negative bacteria		Gentamicin
Escherichia coli (ATCC:10536)	NA	27±1.0
Klebsiella pneumonia (ATCC:10031)	12.3±0.6	25.3 ±0.7
Pseudomonas aeruginosa (ATCC:27853)	12.7±0.6	28±1.0
Gram-positive bacteria		Ampicillin
Staphylococcus aureus (ATCC:13565)	NA	21.3±0.7
Streptococcus mutans (ATCC:25175)	9.3±0.6	28.3±0.7
Fungal phytopathogens		Nystatin
Alternaria alternate (SCUF0000310)	18.3±0.6	21±0.5
Rhizoctonia solani (SCUF0000317)	21.3±0.6	19±0.5
Fusarium oxysporum (SCUF000091)	17.0±1.0	15±0.5

Values are means of three replicates ± standard deviation (SD)

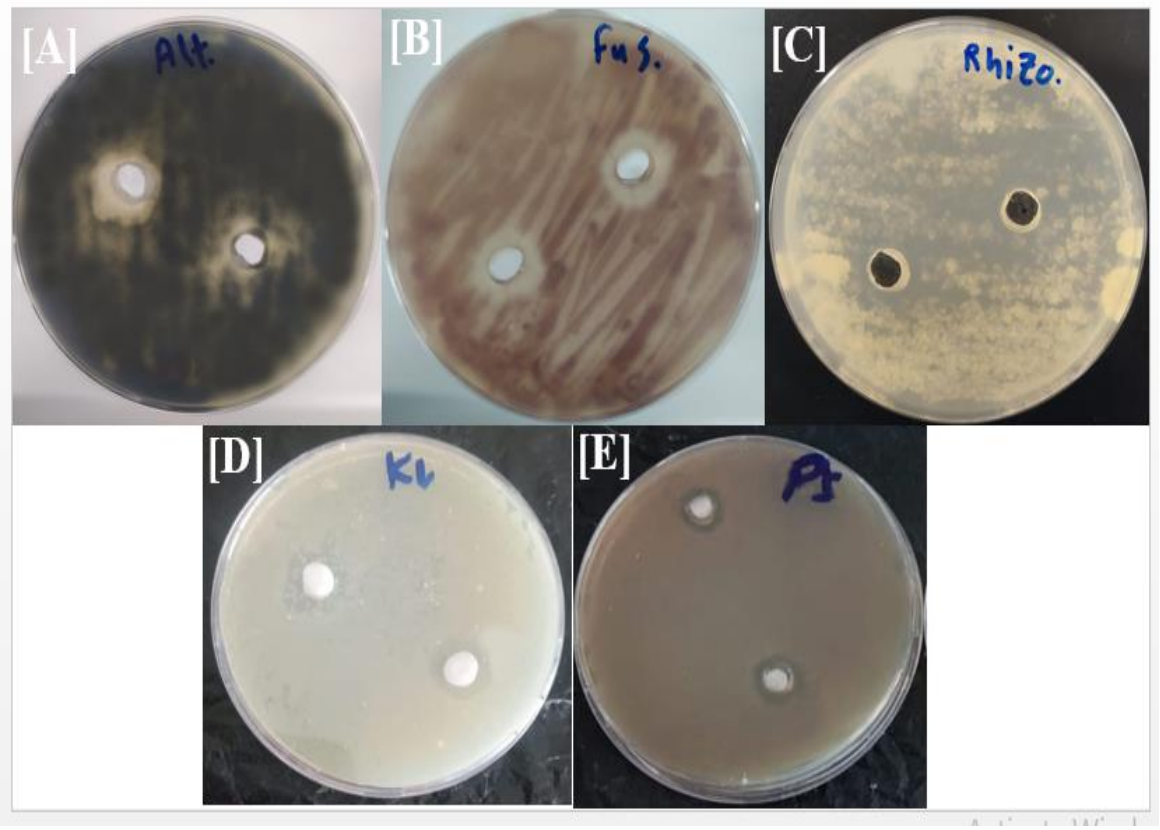

Figure 2. Antimicrobial activities of pure metabolite isolated from *Sphingomonas egypticus* DM against [A] *Alternaria alternate*, [B] *Fusarium oxysporum*, [C] *Rhizoctonia solani*, [D] *Klebsiella pneumonia*, [E] *Pseudomonas aeruginosa*.

Minimum Inhibitory Concentrations (MICs)

According to the study, the growth of pathogenic microorganisms was effectively inhibited by potent secondary metabolite. The pure secondary metabolite exhibited a minimum inhibitory concentration (MIC) range of 125 to 500 µg/mL when tested against various pathogens. The pure metabolite demonstrated the highest efficacy against *Fusarium oxysporum* with a MIC value of 125 µg/mL. At the same time, it exhibited the lowest efficacy against *Klebsiella pneumonia*, indicating a MIC value of 500 µg/mL (Table 3).

Table 3. MICs of pure secondary metabolite recovered from *Sphingomonas egypticus* against the tested organisms.

Sample / Microorganism	MICs (µg/mL)	
	Pure Secondary metabolite	Standard antibiotic
Gram-negative bacteria		Gentamicin
Escherichia coli (ATCC:*10536*)	-	31.25
Klebsiella pneumonia (ATCC:10031)	500	62.5
Pseudomonas aeruginosa (ATCC:27853)	250	125
Gram-positive bacteria		Ampicillin
Staphylococcus aureus (ATCC:13565)	-	62.5
Streptococcus mutans (ATCC:25175)	-	62.5
Fungal phytopathogens		Nystatin
Alternaria alternate (SCUF0000310)	250	25
Rhizoctonia solani (SCUF0000317)	250	22.3
Fusarium oxysporum (SCUF000091)	125	32.4

A recent study examined different metabolites from the same strain, *Sphingomonas,* to determine their Minimum Inhibitory Concentration (MIC) values. The results showed that two metabolites, sattabacin-like and xenocyloin B, had MIC values below 32 µg/mL when isolated from a co-culture of *Paenibacillus* and *Sphingomonas* strains against various clinical strains of *Acinetobacter baumannii* (Qi et al., 2021). This study underscores the significance of developing drugs with lower MIC values, as they would be more cost-effective and productive.

Screening of Lipopeptide *sfp* and PKS Genes

The presence of the lipopeptide 4'-phosphopantetheinyl transferase *sfp* gene in *Sphingomonas egypticus* DM was confirmed through PCR amplification, indicating the strain's antimicrobial activity. Moreover, the results showed that the PKS KS domain was successfully amplified in the DNA template from the rhizospheric strain, as depicted in Figure 3. Subsequent annealing-temperature gradient PCRs were conducted to verify the initial PCR results. The amplified fragments were sequenced to validate that they were indeed the lipopeptide *sfp* gene and PKS KS domain. Sequencing was performed on all unique clones of the appropriate size (approximately 675 bp for the lipopeptide *sfp* gene and 680 bp for the PKS KS domain). A phylogenetic tree was generated to compare the KS domains of various microorganisms. The PKS KS fragments from *Sphingomonas egypticus* DM exhibited the highest similarity to the KS fragments of *Salmonella enenterica* and *Escherichia coli* (Figure 4). Meanwhile, the lipopeptide *sfp* fragments from the same strain revealed sequence similarity to the fragments of *Bacillus sibtilis* (Figure 5). The sequences presented in this study have been deposited in GenBank under the accession numbers OR469907 and OR499756.

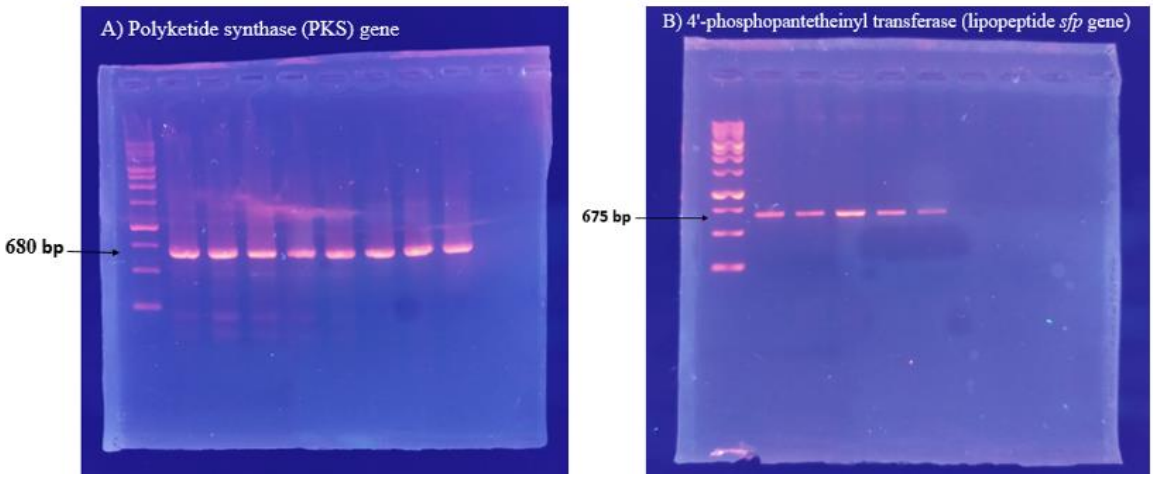

Figure 3. PCR amplification shows polyketide synthase (**A**) and lipopeptide *sfp* (**B**) genes.

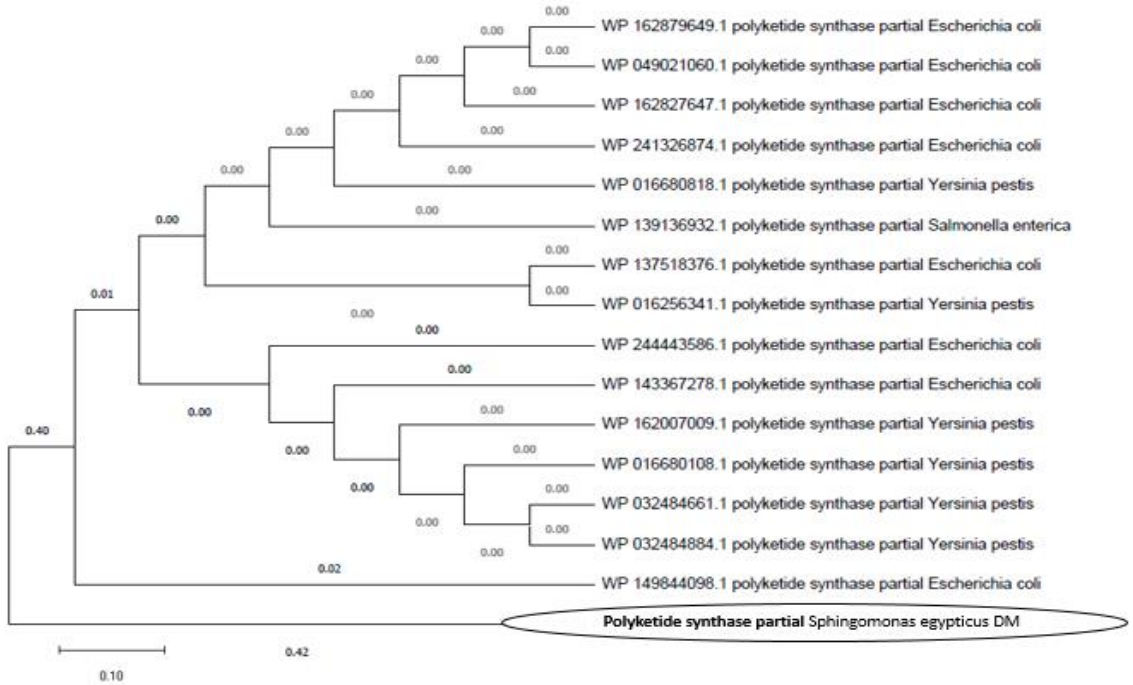

Figure 4. Phylogenetic analysis of KS domain from Sphingomonas egypticus DM compared to other microorganisms' diverse KS fragments

The production of lipopeptides in *Bacillus* spp. is dependent on the activity of a pivotal factor, the 4′-phosphopantetheinyl transferase (PPTase), which is encoded by the *sfp* gene (Jin et al., 2017). Lipopeptides are considered to be major antimicrobial compounds that are grouped under surfactins. It has been discovered that surfactin is a highly effective antibacterial agent through gene amplification screening (Gond et al., 2015). The genome of *Sphingomonas egypticus* DM has confirmed the presence of the antimicrobial surfactin gene (as shown in Figure 3). This exciting discovery suggests that *Sphingomonas egypticus* DM may secrete this antimicrobial lipopeptide in the rhizosphere of *Datura metel* to protect the plant from various pathogens. Other lipopeptides, such as surfactin-like bamylocin A from *Bacillus amyloliquefaciens*, kurstakin from *Bacillus thuringiensis*, maltacines from *Bacillus subtilis*, and polymyxins from *Bacillus polymyxa*, have also been reported (Hagelin et al., 2007; Hathout et al., 2000; Lee et al., 2007; Storm et al., 1977). Polyketide Synthase is a biosynthetic system in microorganisms synthesizing numerous biologically active compounds. To determine whether this system exists in the isolated bioactive bacteria, PCR primers were created to amplify the KS domains of PKS. After screening, the results confirmed the presence of PKS in the bacterial strain. Therefore, this study supports the hypothesis that bioactive bacteria should contain PKS-specific genes. To pinpoint the biosynthetic gene in bioactive bacteria, combining bioactivity screening with secondary metabolite biosynthetic gene screening is crucial while incorporating conserved sequences from other biosynthetic pathways for PCR screening (Zhu et al., 2009).

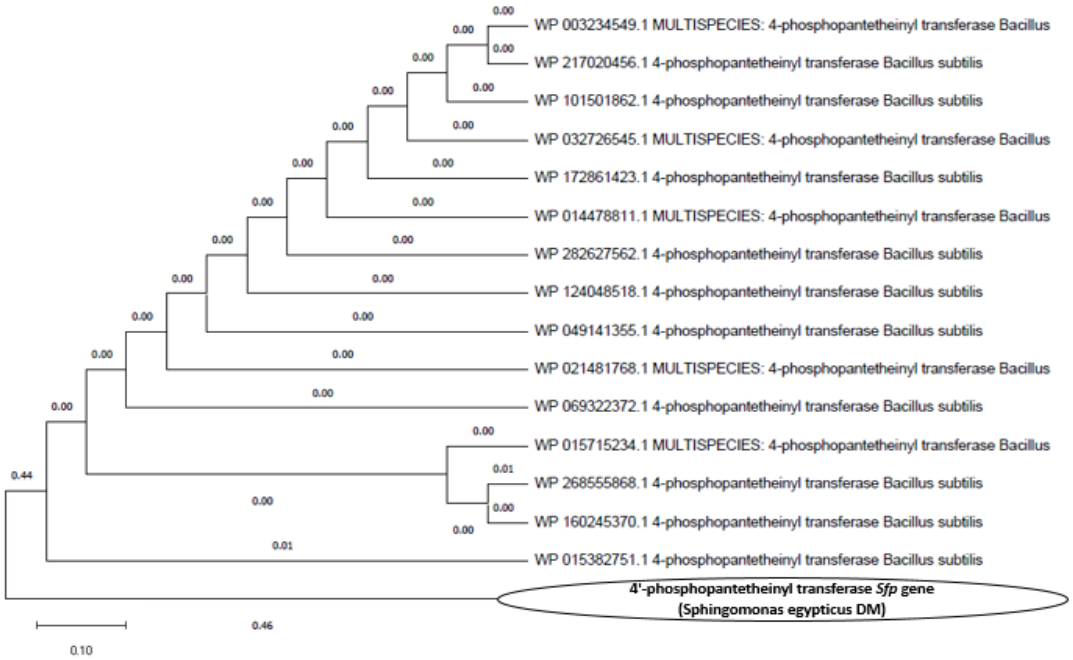

Figure 5. Phylogenetic analysis of lipopeptide *sfp* gene from *Sphingomonas egypticus* DM compared with various lipopeptide *sfp* fragments of *Bacillus subtilis*.

Conclusion

Using various chromatographic methods, we successfully extracted a pure secondary metabolite from the crude extract of rhizospheric *Sphingomonas egypticus* DM. Our findings suggest that the isolated compound exhibits promising biological activity against pathogenic fungi, specifically *Rhizoctonia solani* and *Alternaria alternate*. Notably, this strain demonstrated the lowest minimum inhibitory concentrations (MICs) against *Fusarium oxysporum*. Further PCR amplification and sequencing analysis revealed the presence of PKS and lipopeptide *sfp* genes in the strain. We have deposited the obtained sequences in the NCBI GenBank database under OR469907 and OR499756.

Recommendations

It is necessary to develop various techniques that combine bioactivity screening with screening for genes related to secondary metabolite biosynthesis to determine the factors causing the biological effect.

Scientific Ethics Declaration

The authors declare that the scientific, ethical and legal responsibility of this article published in EPHELS journal belongs to the current authors. The manuscript contains original work, and all authors mutually agree for submission.

Acknowledgements or Notes

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References

Afsharypuor, S., Mostajeran, A., & Mokhtary, R. (1995). Variation of scopolamine and atropine in different parts of Datura metel during development. *Planta Medica*, *61*(4), 383–384.

Atanasov, A. G., Waltenberger, B., Pferschy-Wenzig, E. M., Linder, T., Wawrosch, C., Uhrin, P., Temml, V., Wang, L., Schwaiger, S., Heiss, E. H., Rollinger, J. M., Schuster, D., Breuss, J. M., Bochkov, V., Mihovilovic, M. D., Kopp, B., Bauer, R., Dirsch, V. M., & Stuppner, H. (2015). Discovery and resupply of pharmacologically active plant-derived natural products: A review. *Biotechnology Advances*, *33*(8), 1582–1614.

Bodenhausen, N., Horton, M. W., & Bergelson, J. (2013). Bacterial communities associated with the leaves and the roots of Arabidopsis thaliana. *PLoS One*, *8*(2).

Buedenbender, L., Carroll, A. R., Ekins, M., & Kurtböke, D. I. (2017). Taxonomic and metabolite diversity of actinomycetes associated with three Australian ascidians. *Diversity*, *9*(4), 53.

Buell, C. R. (2002). Interactions between Xanthomonas Species and Arabidopsis thaliana. *The Arabidopsis Book*, *1*, e0031.

Busse, H. J., Denner, E. B. M., Buczolits, S., Salkinoja-Salonen, M., Bennasar, A., & Kämpfer, P. (2003). Sphingomonas aurantiaca sp. nov., Sphingomonas aerolata sp. nov. and Sphingomonas faeni sp. nov., air- and dustborne and Antarctic, orange-pigmented, psychrotolerant bacteria, and emended description of the genus Sphingomonas. *International Journal of Systematic and Evolutionary Microbiology*, *53*(5), 1253–1260.

Chen, Jogler, M., Rohde, M., Klenk, H. P., Busse, H. J., Tindall, B. J., & Overmann, J. (2012). Reclassification and emended description of Caulobacter leidyi as Sphingomonas leidyi comb. nov., and emendation of the genus Sphingomonas. *International Journal of Systematic and Evolutionary Microbiology*, *62*(12), 2835–2843.

Chen, L., Wang, X. N., Fu, C. M., & Wang, G. Y. (2019). Phylogenetic Analysis and Screening of Antimicrobial and Antiproliferative Activities of Culturable Bacteria Associated with the Ascidian Styela clava from the Yellow Sea, China. *BioMed Research International Article ID 7851251* https://doi.org/10.1155/2019/7851251

Chudáčková, E., Bergerová, T., Fajfrlík, K., Červená, D., Urbášková, P., Empel, J., Gniadkowski, M., & Hrabák, J. (2010). Carbapenem-nonsusceptible strains of Klebsiella pneumoniae producing SHV-5 and/or DHA-1 β-lactamases in a Czech hospital. *FEMS Microbiology Letters*, *309*(1), 62–70.

Chung, E. J., Jo, E. J., Yoon, H. S., Song, G. C., Jeon, C. O., & Chung, Y. R. (2011). Sphingomonas oryziterrae sp. nov. and sphingomonas jinjuensis sp. nov. isolated from rhizosphere soil of rice (oryza sativa l.). *International Journal of Systematic and Evolutionary Microbiology*, *61*(10), 2389–2394.

Davidson, S. K., Allen, S. W., Lim, G. E., Anderson, C. M., & Haygood, M. G. (2001). Evidence for the Biosynthesis of Bryostatins by the Bacterial Symbiont "Candidatus Endobugula sertula" of the Bryozoan Bugula neritina. *Applied and Environmental Microbiology*, *67*(10), 4531–4537.

De Medici, D., Croci, L., Delibato, E., Di Pasquale, S., Filetici, E., & Toti, L. (2003). Evaluation of DNA extraction methods for use in combination with SYBR green I real-time PCR to detect Salmonella enterica serotype Enteritidis in poultry. *Applied and Environmental Microbiology*, *69*(6), 3456–3461.

Fegatella, F., & Cavicchioli, R. (2000). Physiological responses to starvation in the marine oligotrophic ultramicrobacterium Sphingomonas sp. strain RB2256. *Applied and Environmental Microbiology*, *66*(5), 2037–2044.

Feng, G. Da, Yang, S. Z., Xiong, X., Li, H. P., & Zhu, H. H. (2017). Sphingomonas spermidinifaciens sp. Nov., a novel bacterium containing spermidine as the major polyamine, isolated from an abandoned lead–zinc mine and emended descriptions of the genus Sphingomonas and the species Sphingomonas yantingensis and Sphingomona. *International Journal of Systematic and Evolutionary Microbiology*, *67*(7), 2160–2165.

Gond, S. K., Bergen, M. S., Torres, M. S., & White, J. F. (2015). Endophytic Bacillus spp. produce antifungal lipopeptides and induce host defence gene expression in maize. *Microbiological Research*, *172*, 79–87.

Hagelin, G., Indrevoll, B., & Hoeg-Jensen, T. (2007). Use of synthetic analogues in confirmation of structure of the peptide antibiotics Maltacines. *International Journal of Mass Spectrometry*, *268*(2–3), 254–264.

Hamed, A., Abdel-Razek, A. S., Araby, M., Abu-Elghait, M., El-Hosari, D. G., Frese, M., Soliman, H. S. M., Stammler, H. G., Sewald, N., & Shaaban, M. (2021). Meleagrin from marine fungus Emericella dentata Nq45: crystal structure and diverse biological activity studies. *Natural Product Research*, *35*(21), 3830–3838.

Hathout, Y., Ho, Y. P., Ryzhov, V., Demirev, P., & Fenselau, C. (2000). Kurstakins: A new class of lipopeptides isolated from Bacillus thuringiensis. *Journal of Natural Products*, *63*(11), 1492–1496.

Hildebrand, M., Waggoner, L. E., Lim, G. E., Sharp, K. H., Ridley, C. P., & Haygood, M. G. (2004). Approaches to identify, clone, and express symbiont bioactive metabolite genes. *Natural Product*

Reports, *21*(1), 122–142.

Huang, J., Hu, R., Pray, C., Qiao, F., & Rozelle, S. (2003). Biotechnology as an alternative to chemical pesticides: A case study of Bt cotton in China. *Agricultural Economics*, *29*(1), 55–67.

Innerebner, G., Knief, C., & Vorholt, J. A. (2011). Protection of Arabidopsis thaliana against leaf-pathogenic Pseudomonas syringae by Sphingomonas strains in a controlled model system. *Applied and Environmental Microbiology*, *77*(10), 3202–3210.

Islam, T., Ara, I., Islam, T., Sah, P. K., Almeida, R. S. de, Matias, E. F. F., Ramalho, C. L. G., Coutinho, H. D. M., & Islam, M. T. (2023). Ethnobotanical uses and phytochemical, biological, and toxicological profiles of Datura metel L.: A review. *Current Research in Toxicology*, *4*, 100106.

Ji, P., & Wilson, M. (2002). Assessment of the importance of similarity in carbon source utilization profiles between the biological control agent and the pathogen in biological control of bacterial speck of tomato. *Applied and Environmental Microbiology*, *68*(9), 4383–4389.

Jin, P., Wang, H., Liu, W., & Miao, W. (2017). Characterization of lpaH2 gene corresponding to lipopeptide synthesis in Bacillus amyloliquefaciens HAB-2. *BMC Microbiology*, *17*(1), 1–10.

Kennedy, R. K., Naik, P. R., Veena, V., Lakshmi, B. S., Lakshmi, P., Krishna, R., & Sakthivel, N. (2015). 5-Methyl phenazine-1-carboxylic acid: A novel bioactive metabolite by a rhizosphere soil bacterium that exhibits potent antimicrobial and anticancer activities. *Chemico-Biological Interactions*, *231*, 71–82.

Kumar, S., Tamura, K., Jakobsen, I. B., & Nei, M. (2002). MEGA2: Molecular evolutionary genetics analysis software. *Bioinformatics*, *17*(12), 1244–1245.

Lang, S. (2002). Biological amphiphiles (microbial biosurfactants). *Current Opinion in Colloid and Interface Science*, *7*(1–2), 12–20.

Lee, S. C., Kim, S. H., Park, I. H., Chung, S. Y., & Choi, Y. L. (2007). Isolation and structural analysis of bamylocin A, novel lipopeptide from Bacillus amyloliquefaciens LP03 having antagonistic and crude oil-emulsifying activity. *Archives of Microbiology*, *188*(4), 307–312.

Leys, N. M. E. J., Ryngaert, A., Bastiaens, L., Verstraete, W., Top, E. M., & Springael, D. (2004). Occurrence and Phylogenetic Diversity of Sphingomonas Strains in Soils Contaminated with Polycyclic Aromatic Hydrocarbons. *Applied and Environmental Microbiology*, *70*(4), 1944–1955.

Linch, A. L., Hendrickson, E. R., Katz, M., Martin, J. R., Nelson, G. O., Pattison, J. N., Vander Kolk, A. L., & Weidner, R. B. (1973). Thin layer chromatography. *Health Laboratory Science*, *10*(2), 141–152.

Newman, D. J., & Cragg, G. M. (2016). Natural Products as Sources of New Drugs from 1981 to 2014. *Journal of Natural Products*, *79*(3), 629–661.

Pastor, N., Carlier, E., Andrés, J., Rosas, S. B., & Rovera, M. (2012). Characterization of rhizosphere bacteria for control of phytopathogenic fungi of tomato. *Journal of Environmental Management*, *95*(SUPPL.)332–337.

Peypoux, F., Guinand, M., Michel, G., Delcambe, L., Das, B. C., & Lederer, E. (1978). Structure of Iturine a, a peptidolipid antibiotic from Bacillus subtilis. *Biochemistry*, *17*(19), 3992–3996.

Philippot, L., Raaijmakers, J. M., Lemanceau, P., & Van Der Putten, W. H. (2013). Going back to the roots: The microbial ecology of the rhizosphere. *Nature Reviews Microbiology*, *11*(11), 789–799.

Piel, J., Hui, D., Wen, G., Butzke, D., Platzer, M., Fusetani, N., & Matsunaga, S. (2004). Antitumor polyketide biosynthesis by an uncultivated bacterial symbiont of the marine sponge Theonella swinhoei. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(46), 16222–16227.

Qi, S. S., Bogdanov, A., Cnockaert, M., Acar, T., Ranty-Roby, S., Coenye, T., Vandamme, P., König, G. M., Crüsemann, M., & Carlier, A. (2021). Induction of antibiotic specialized metabolism by co-culturing in a collection of phyllosphere bacteria. *Environmental Microbiology*, *23*(4), 2132–2151.

Raaijmakers, J. M., Paulitz, T. C., Steinberg, C., Alabouvette, C., & Moënne-Loccoz, Y. (2009). The rhizosphere: a playground and battlefield for soilborne pathogens and beneficial microorganisms. *Plant and Soil*, *321*(1), 341–361.

Radić, N., & Štrukelj, B. (2012). Endophytic fungi - The treasure chest of antibacterial substances. *Phytomedicine*, *19*(14), 1270–1284.

Ratan, L., Meenu, B., Anju, D., & Arun, N. (2011). Morpo-anatomical and physicochemical studies of dried seeds of Datura fastuosa Linn. *International Research Journal of Pharmacy*, *2*(3), 208–212.

Rivas, R., Abril, A., Trujillo, M. E., & Velázquez, E. (2004). Sphingomonas phyllosphaerae sp. nov., from the phyllosphere of Acacia caven in Argentina. *International Journal of Systematic and Evolutionary Microbiology*, *54*(6), 2147–2150.

Schmidt, E. W., Nelson, J. T., Rasko, D. A., Sudek, S., Eisen, J. A., Haygood, M. G., & Ravel, J. (2005). Schmidt EW 2005 Patellamides A und C from Prochloron didemni.pdf. *Pnas*, *102*(20), 7315–7320.

Scott, A. C. (1989). Laboratory control of antimicrobial therapy. *Mackie and MacCartney Practical Medical Microbiology*, *2*, 161–181.

Seo, J. S., Keum, Y. S., & Li, Q. X. (2009). Bacterial degradation of aromatic compounds. In *International*

Journal of Environmental Research and Public Health, *6*(1).,278-309.
Shen, L., Liu, P., An, M., Liang, R., He, X., & Zhao, G. (2022). Sphingomonas quercus sp. nov., Isolated from Rhizosphere Soil of Quercus mongolica. *Current Microbiology*, *79*(4), 1–7.
Springer-Verlag. (1984). Spray Reagents for the thin layer chromatography of drug extracts. *Bookmatter Plant Drug Analysis*, *1*, 299–320. Retrieved from http://delloyd.50megs.com/spray_reagents.html#C
Staunton, J., & Weissman, K. J. (2001). Polyketide biosynthesis: A millennium review. *Natural Product Reports*, *18*(4), 380–416.
Storm, D. R., Rosenthal, K. S., & Swanson, P. E. (1977). Polymyxin and related peptide antibiotics. *Annual Review of Biochemistry*, *46*, 723–763.
Takeuchi, M., Hamana, K., & Hiraishi, A. (2001). Proposal of the genus Sphingomonas sensu stricto and three new genera. *International Journal of Systematic and Evolutionary Microbiology*, *51*(4), 1405–1417.
Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., & Higgins, D. G. (1997). The CLUSTAL X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, *25*(24), 4876–4882.
Vanittanakom, N., Loeffler, W., Koch, U., & Jung, G. (1986). Fengycin-a novel antifungal lipopeptide antibiotic produced by Bacillus Subtilis F-29-3. *Journal of Antibiotics*, *39*(7), 888–901.
Xun-Chao, C., Hui, L., Ya-Rong, X., & Chang-Hong, L. (2013). Study of endophytic Bacillus amyloliquefaciens CC09 and its antifungal cyclic lipopeptides. *Journal of Applied Biology and Biotechnology*, *1*(1), 1–5.
Yabuuchi, E., Kosako, Y., Fujiwara, N., Naka, T., Matsunaga, I., Ogura, H., & Kobayashi, K. (2002). Emendation of the genus Sphingomonas Yabuuchi et al. 1990 and junior objective synonymy of the species of three genera, Sphingobium, Novosphingobium and Sphingopyxis, in conjunction with Blastomonas ursincola. *International Journal of Systematic and Evolutionary Microbiology*, *52*(5), 1485–1496.
Zhang, H., Xu, L., Zhang, J. X., & Sun, J. Q. (2020). Sphingomonas suaedae sp. Nov., a chitin-degrading strain isolated from rhizosphere soil of suaeda salsa. *International Journal of Systematic and Evolutionary Microbiology*, *70*(6), 3816–3823.
Zhou, L., Song, C., Li, Z., & Kuipers, O. P. (2021). Antimicrobial activity screening of rhizosphere soil bacteria from tomato and genome-based analysis of their antimicrobial biosynthetic potential. *BMC Genomics*, *22*(1), 1–14.
Zhu, L., Si, M., Li, C., Xin, K., Chen, C., Shi, X., Huang, R., Zhao, L., Shen, X., & Zhang, L. (2015). Sphingomonas gei sp. nov., isolated from roots of Geum aleppicum. *International Journal of Systematic and Evolutionary Microbiology*, *65*, 1160–1166.
Zhu, P., Zheng, Y., You, Y., Yan, X., & Shao, J. (2009). Molecular phylogeny and modular structure of hybrid NRPS/PKS gene fragment of Pseudoalteromonas sp. NJ6-3-2 isolated from marine sponge Hymeniacidon perleve. *Journal of Microbiology and Biotechnology*, *19*(3), 229–237.

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Determination of Some miRNA Expression Levels in Chronic Lymphoid Leukemia Patients

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Abstract: Chronic lymphoid leukemia (CLL) is a type of cancer that occurs as a result of the accumulation of morphologically small, mature-looking lymphocytes. CLL is the most common type of leukemia in Western countries, where it accounts for 30% of all leukemias. Accounts for only 10% of all leukemias in Asian populations. MicroRNAs (miRNAs) are non-protein-coding single-stranded RNA molecules approximately 18-25 nucleotides long, forming a class of endogenous small RNAs. Research has shown that microRNAs can function as oncogenes or tumor suppressors in CLL. Although the expression levels of microRNA 133a and microRNA 452 have been determined in many cancers, including lung, prostate and colon cancer, expression levels in CLL patients have not been determined. Therefore, in our study, the expression levels of miRNA 133a and miRNA 452 in CLL patients will be calculated quantitatively using the Real-Time PCR method. As for the method steps, in the first stage, whole blood samples will be taken. miRNA will be isolated from the whole blood samples taken, cDNA will be synthesized from the miRNA samples, and finally, expression levels will be determined with the Real-Time PCR method using miRNA 133a, miRNA452 specific primers and U6 primer as the reference gene. The data obtained will be analyzed and interpreted with the SPSS package program. This study will be conducted to determine whether these two miRNAs can guide early diagnosis and diagnosis in CLL patients and to provide preliminary information to clinicians and contribute to the literature on this subject.

Keywords: CLL, miRNA, Real-time PCR, Expression

Introduction

Cancer is a disease at the cellular level. Clinically cancer; It is expressed as a condition that includes nearly a hundred complex diseases that exhibit different behaviors depending on the cell type from which they originate. Cancers; They vary according to their age of onset, growth rate, spread, stage, and treatment response. On the other hand, all cancer types have common characteristics at the molecular level that bring them together under a single title (Klug et al., 2011).

Cancer is one of the deaths with known causes both in the world and in our country; It is an important public health problem as it is the second cause of death after heart and circulatory system diseases. At the same time it is a chronic disease that is increasing worldwide and causing significant material, spiritual, social and economic losses in societies. Cancer causes the death of 8.2 million people and infects 14 million people every year in the world; It affects all people (Ergin et al., 2019). Leukemia, one of these cancer types, is a type of cancer caused by the abnormal proliferation of developing leukocytes. It is divided into two as acute or chronic according to

the rate of proliferation, and myeloid or lymphoid according to the cell of origin. While acute leukemias are seen in children, chronic leukemias are seen more in adults (Chennamadhavuni et al., 2023).

Chronic lymphoid leukemia (CLL) is a disease characterized by clonal proliferation and accumulation of neoplastic B lymphocytes in the peripheral blood, spleen, bone marrow and lymph nodes (Rozman & Montserrat, 1995). CLL is the most common type of leukemia in Western countries, accounting for approximately 25-30% of all leukemias. The majority of patients are asymptomatic, so it is difficult to determine its actual frequency. The incidence rate is approximately 2-6 new cases per 100,000 people and is stated to increase with age, reaching 12.8/100,000/year at the age of 65, which is the average age of diagnosis. Although the average age of diagnosis is 65, in recent years, it has been reported that one-third of new cases are also seen in young individuals under the age of 55. It has been stated that chronic lymphoid leukemia disease is more common in men than in women and the gender ratio is approximately 1.5-2:1 (Ghia et al., 2007).

Although the etiology of CLL is not fully known, there has been no apparent relationship between its occurrence and any known environmental factors such as ionizing radiation, chemicals and drugs. Epidemiological evidence indicates that genetic factors and familial predisposition are important in the pathogenesis of the disease (Kalil & Cheson, 1999).

CLL can be found in two forms: aggressive and slowly progressing disease. While some CLL patients do not need treatment for many years, patients with aggressive form CLL need urgent treatment. Furthermore slow CLL can progress to an aggressive form, so it is important to find new markers for specific and early diagnosis of CLL prognosis and staging (Balatti et al., 2016). Two different staging systems used in CLL are Rai and Binet staging systems. When both staging systems are examined, it can be seen that both have some deficiency. Many parameters that determine prognosis in CLL have been defined. These parameters; The doubling time of the leukocyte count, mutation status in the variable gene of the immunoglobulin heavy chain(IgVH), CLL cells CD38 and (ZAP70) expressions, Lactate Dehydrogenase(LDH) level, serum ß2-microglobulin level, serum thymidine kinase activity can be counted(Soysal, 2013).

CLL is a type of leukemia that occurs as a result of malignant transformation of mature B-lymphocytes. As 90% of CLL cells don't have proliferation capacity, they are in G0/G1 cell transformation. For this reason, it is suggested that CLL disease is caused by the disruption of the apoptosis mechanism, but not by excessive proliferation of B-cells. Studies have shown that the proto-oncogene bcl-2(B-cell lymphoma-2) is overexpressed in B-cell CLL. The Bcl-2 family, which includes pro-apoptotic (bax, bok, and bak) and anti-apoptotic (bcl-2, bcl-w, bcl-xl, and mcl-1) proteins, has been implicated as important regulators of apoptosis. Among the 11 proteins involved in the control of apoptosis, proliferation and differentiation, bcl-2 is stated to be one of the most important proteins that determine the pathogenesis of CLL and ensures the longevity of the relevant cells (Alkan, 2006; Del Principe et al., 2016).

CLL is divided into two subgroups depending on the presence or absence of somatic mutations in the variable genes of the immunoglobulin heavy chain locus (IgVH). IgVH mutation status has different biological and clinical significance: Compared to cases with unmutated IgVH, cases with mutated IgVH show stable disease and longer survival. Mutation of TP53, a tumor suppressor, is associated with chromosome 17p13 deletion, and they generally have poor prognosis and resistance to treatment (Mraz et al., 2009). Like the TP53 mutation, mutations in many genes including SF3B1, NOTCH1, MYD88, ATM, SAMHD1, NRAS and BIRC3 have been stated to be associated with CLL prognosis (Kaur et al., 2020).

Cytogenetic anomalies seen in CLL are important in determining prognosis and treatment options during diagnosis. There are no single specific cytogenetic abnormalities unique to CLL. Detection of chromosome abnormalities has increased thanks to new methods such as Fluorescent in situ hybridization (FISH). The most common cytogenetic abnormalities are 13q14 deletion (-51%), 11q22-23 deletion (17-20%), trisomy 12 (15%) and 17p13 deletion. While cases with 13q14 deletion show good prognosis, cases with trisomy 12 constitute the intermediate risk group. The presence of deletions 11q and 17p has been associated with non-response to chemotherapy, short survival and poor prognosis(Soysal, 2013; Kalil & Cheson, 1999)

What is miRNA? and miRNA CLL Relationship

miRNAs were first identified as genes that play a regulatory role in developmental timing events in a model organism, C. elegans (Kato & Slack, 2008). miRNAs are small non-protein-coding RNA molecules approximately 18-25 nucleotides long. These molecules influence many biological processes, including cellular

proliferation, differentiation, and apoptosis, and play important roles in normal development, physiology, and disease (Çelik et al., 2013; Chandrasekaran et al., 2019). Apart from these features, more than half of miRNA molecules have been found to be located in cancer-related gene regions or fragile regions in the human genome. In cancer development, miRNAs may act as oncogenes or tumor suppressors in relation to the mRNAs they target, therefore miRNAs appear to be regulators of tumor progression, metastasis and invasion. (Çelik et al., 2013; Saydam et al., 2011). Revealing new mechanisms and relationships will also contribute to the development of diagnostic and treatment methods.

The first study revealing a miRNA relationship with cancer was the study conducted by Calin et al. They found that this region, located on chromosome 13q14, a region that is deleted in approximately half of B-cell chronic lymphoid leukemias (B-CLL), contains miR15 and miR16 genes. İt was determined that miR15 and miR16 are located within a 30-kb region of loss in CLL. They then studied miR15 and miR16 expression in blood samples from CLL patients. Looking at the results, it was stated that the expression of these miRNAs is reduced or absent in approximately 68% of CLL patients. The emergence of these expression differences has further strengthened the roles of miRNAs in cancer pathogenesis. (Calin et al., 2002; Sassen et al., 2008). After this study, studies on cancer and miRNA have accelerated.

In their study to create miRNA expression profiles in 94 CLL patients, Calin et al. reported 13 miRNAs that correlated with CLL. (Calin et al., 2005) In the study conducted by Farzadfard et al. to investigate the expression of a group of miRNAs (miRNA 30d, 25-3p, 19a-3p, 133b, 451a, 145 and 144) in CLL patients; They used the RT-PCR method on serum samples of CLL patients. The results showed that these miRNAs were dysregulated in CLL samples compared to healthy controls. They also concluded that miR-19a-3p and miR-25-3p were significantly increased in CLL patients and that they may play important roles in the pathogenicity of CLL (Farzadfard et al., 2020). In their study by Khalifa et al. to evaluate the prognostic value of members of the miRNA 17-92 gene cluster in Egyptian CLL patients, expression levels of miR17-92 gene cluster members, including miR17, miR18a, miR19a, miR19b-1, miR20a and miR92a-1, were evaluated by qRT-PCR. Among other research, they used serum LDH, serum β2 microglobulin (β2M), CD38 and ZAP70 expression by flow cytometry, fluorescence in situ hybridization (FISH) and imaging studies for 17p deletion. Overexpression of the entire cluster was detected in Egyptian CLL patients, and significant associations were found between the miR17-92 gene cluster and various parameters. They concluded that although miR18a, miR19b-1 and miR92a-1 showed negative prognostic value, miR17 may be a good prognostic marker and those with high miR19a expression showed good overall survival compared to those with low expression(Khalifa et al., 2021) Rahimi et al evaluated the expression of miR-32-5p, miR-98-5p, and miR-374b-5p using the Real-Time PCR method in samples from Kermanshah province, Iran and also investigated the signaling pathways regulated by the examined miRs. It showed that there was a significant decrease in the expression of miR-32-5p, miR-98-5p and miR-374-5p in CLL patients at the time of diagnosis and before receiving any treatment. The decreased expression of these miRNAs suggested that they have a tumor suppressor role in CLL. They concluded that these significant changes occurring in the early stages of the disease may also make them candidates as potential biomarkers for early diagnosis of CLL(Rahimi et al., 2021).

Many studies have been put forward for the early diagnosis and treatment of CLL and studies are still ongoing, we have only mentioned a few of them above. As a result of these studies, new information is revealed that will shed light on this disease. More detailed studies aimed at understanding the roles of miRNAs in the development and progression of the disease in CLL patients and identifying new miRNA genes may lead to more accurate diagnoses, prognoses and therapeutic applications in the treatment of CLL disease. Although miRNA133a and miRNA452 have been investigated in other types of cancer, there is no study on their expression levels in CLL patients. For this purpose, we aim to determine miRNA133a and miRNA 452 expression levels in CLL patients by Real-Time PCR method.

Considering the relationship of miRNA133a and miRNA452 with other types of cancer; miRNA 133 has been described as one of the most studied and best characterized miRNAs to date. miRNA 133 has 3 known genes in the human genome; miRNA-133a-1, miRNA-133a-2 and miRNA-133b. miRNA 133a has been associated with cancer and has been implicated in breast cancer, colorectal cancer, lung carcinoma, bladder cancer, prostate cancer, etc. It has been identified as a key factor in the development of cancer, including (Yu et al., 2014; Wang, 2020) miRNA 452 is a recently identified cancer-associated miRNA and is divided into two subtypes: miR-452-5p and miR-452-3p. Abnormal expression of MiR-452 has been identified in many types of cancer, including kidney cancer, prostate cancer, non-small cell lung cancer, osteosarcoma, breast cancer and colorectal cancer, and it has been stated that it plays two different roles as a potential tumor suppressor gene and oncogene (Karimi Dermani et al., 2023).

Conclusion

This study will be conducted to determine whether these two miRNAs can guide early diagnosis and diagnosis in CLL patients and to provide preliminary information to clinicians and contribute to the literature on this subject.

Scientific Ethics Declaration

The authors declare that the scientific ethical and legal responsibility of this article published in EPHELS journal belongs to the authors.

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References

Alkan, S. (2006). *Kronik lenfositik losemi (KLL): Patogenezi ve biyolojisi*. Retrieved from http://www.thd.org.tr/thdData/userfiles/file/kroniklenfositik.pdf

Çelik, D. A., Koşar, P. A., & Özçelik, N. (2013). MikroRNA lar ve kanser ile iliþkisi. *SDÜ Tıp Fakültesi Dergisi*, *20*(3), 121-127.

Balatti, V., Acunzo, M., Pekarky, Y., & Croce, C. M. (2016). Novel mechanisms of regulation of miRNAs in CLL. *Trends in cancer*, *2*(3), 134–143.

Calin, G. A., Dumitru, C. D., Shimizu, M., Bichi, R., Zupo, S., Noch, E., Aldler, H., Rattan, S., Keating, M., Rai, K., Rassenti, L., Kipps, T., Negrini, M., Bullrich, F., & Croce, C. M. (2002). Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(24), 15524–15529.

Calin, G. A., Ferracin, M., Cimmino, A., Di Leva, G., Shimizu, M., Wojcik, S. E., Iorio, M. V., Visone, R., Sever, N. I., Fabbri, M., Iuliano, R., Palumbo, T., Pichiorri, F., Roldo, C., Garzon, R., Sevignani, C., Rassenti, L., Alder, H., Volinia, S., Liu, C. G., … Croce, C. M. (2005). A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. *The New England journal of medicine*, *353*(17), 1793–1801.

Chandrasekaran, A. R., MacIsaac, M., Dey, P., Levchenko, O., Zhou, L., Andres, M., Dey, B. K., & Halvorsen, K. (2019). Cellular microRNA detection with miRacles: microRNA- activated conditional looping of engineered switches. *Science advances*, *5*(3), eaau9443.

Chennamadhavuni, A., Lyengar, V., Mukkamalla, S. K. R., & Shimanovsky, A. (2023). *Leukemia*. StatPearls Publishing.

Del Principe, M. I., Dal Bo, M., Bittolo, T., Buccisano, F., Rossi, F. M., Zucchetto, A., Rossi, D., Bomben, R., Maurillo, L., Cefalo, M., De Santis, G., Venditti, A., Gaidano, G., Amadori, S., de Fabritiis, P., Gattei, V., & Del Poeta, G. (2016). Clinical significance of bax/bcl-2 ratio in chronic lymphocytic leukemia. *Haematologica*, *101*(1), 77-85.

Ergin, A., Özdilek, R., & Dutucu, N. (2019). 2012-2017 yılları arasında kadınlarda görülen kanser türleri ve dağılımları: bir üniversite hastanesi örneği. *Kadın Sağlığı Hemşireliği Dergisi*, *5*(1), 1-21.

Farzadfard, E., Kalantari, T., & Tamaddon, G. (2020). Serum expression of seven MicroRNAs in chronic lymphocytic leukemia patients. *Journal of Blood Medicine*, *11*, 97–102.

Ghia, P., Ferreri, A. M., & Caligaris-Cappio, F. (2007). Chronic lymphocytic leukemia. *Critical reviews in oncology/hematology*, *64*(3), 234–246.

Kalil, N., & Cheson, B. D. (1999). Chronic lymphocytic leukemia. *The Oncologist*, *4*(5), 352–369.

Karimi Dermani, F., Datta, I., & Gholamzadeh Khoei, S. (2023). MicroRNA-452: A double-edged sword in multiple human cancers. *Clinical & translational oncology: Official publication of the Federation of Spanish Oncology Societies and of the National Cancer Institute of Mexico*, *25*(5), 1189–1206.

Kaur, G., Ruhela, V., Rani, L., Gupta, A., Sriram, K., Gogia, A., Sharma, A., Kumar, L., & Gupta, R. (2020). RNA-Seq profiling of deregulated miRs in CLL and their impact on clinical outcome. *Blood cancer journal*, *10*(1), 6.

Kato, M., & Slack, F. J. (2008). microRNAs: small molecules with big roles - C. elegans to human cancer. *Biology of the cell*, *100*(2), 71–81.

Khalifa, M. M., Zaki, N. E., Nazier, A. A., Moussa, M. A., Haleem, R. A., Rabie, M. A., & Mansour, A. R. (2021). Prognostic significance of microRNA 17-92 cluster expression in Egyptian chronic lymphocytic leukemia patients. *Journal of the Egyptian National Cancer Institute*, *33*(1), 37.

Klug, W. S., Cummings, M. R., & Spencer, C.A. (2011). *Genetik kavramlar*. Ankara: Palme Yayıncılık.

Mraz, M., Malinova, K., Kotaskova, J., Pavlova, S., Tichy, B., Malcikova, J., Stano Kozubik, K., Smardova, J., Brychtova, Y., Doubek, M., Trbusek, M., Mayer, J., & Pospisilova, S. (2009). miR-34a, miR-29c and miR-17-5p are downregulated in CLL patients with TP53 abnormalities. *Leukemia*, *23*(6), 1159–1163.

Rahimi, Z., Ghorbani, Z., Motamed, H., & Jalilian, N. (2021). Aberrant expression profile of miR-32, miR-98 and miR-374 in chronic lymphocytic leukemia. *Leukemia research*, *111*, 106691.

Rozman, C., & Montserrat, E. (1995). Chronic lymphocytic leukemia. *The New England journal of medicine*, *333*(16), 1052–1057.

Sassen, S., Miska, E. A., & Caldas, C. (2008). MicroRNA: implications for cancer. *Virchows Archiv : an İnternational Journal of Pathology*, *452*(1), 1–10.

Saydam, F., Değirmeci, İ., Güneş, H. V. (2011). MikroRNA' lar ve Kanser. *Dicle Tıp Dergisi, 38*(1), 113-120.

Soysal, T. (2013). Kronik Lenfositik Lösemi-Küçük Lenfositik Lenfoma. Türk Hematoloji Derneği. HematoLog, 3: 253-270.

Wang, Z. (2020). Diagnostic performance for declined microRNA-133a in pancreatic cancer. *Journal of cellular biochemistry*, *121*(8-9), 3882–3886.

Yu, H., Lu, Y., Li, Z., & Wang, Q. (2014). MicroRNA-133: Expression, function and therapeutic potential in muscle diseases and cancer. *Current drug targets*, *15*(9), 817–828.

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Prevalence of HCC among Living Donor Liver Transplant Recipients and Effect on Outcome Georgian Experience

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Abstract: Liver transplantation (LT) may be the best curative treatment that offers a chance of cure for the tumor and the underlying cirrhosis by complete extirpation of both. Hepatocellular Carcinoma (HCC) is a formidable global health challenge, representing one of the most prevalent primary liver malignancies with increasing incidence rates worldwide. It sets the stage for a comprehensive exploration of how LDLT has redefined the paradigms of treatment for HCC and contributed to enhanced patient outcomes. To answer the question of this paper we used cross sectional study design among 84 Living Donor Liver Transplant Recipients. After collecting data analyzing patient's medical cards we have found that out of 84 recipients 12 (11%) had a Hepatocellular Carcinoma, out of this 8 (6.7%) recipients dead and 4 (3.3%) is alive. We may have challenges such as: Diagnostic Challenges, Treatment Options, and Selection Criteria for LDLT in HCC. The relationship between HCC and LDLT is a dynamic and evolving field. LDLT has significantly expanded the therapeutic options for selected HCC patients, offering hope for improved outcomes. However, the intricate interplay between patient selection, surgical techniques, and post-transplant care underscores the importance of a multidisciplinary approach to HCC management. Ongoing research and advances in both HCC treatment and LDLT continue to shape the landscape of this complex disease and transplantation strategy.

Keywords: Transplantation, Hepatocellular Carcinoma, Outcome

Introduction

Liver transplantation (LT) may be the best curative treatment that offers a chance of cure for the tumor and the underlying cirrhosis by complete extirpation of both. (Azzam, 2015). Liver cancer remains a global health challenge, with an estimated incidence of >1 million cases by 2025. Hepatocellular carcinoma (HCC) is the most common form of liver cancer and accounts for ~90% of cases. Infection by hepatitis B virus and hepatitis C virus are the main risk factors for HCC development, although non-alcoholic steatohepatitis associated with metabolic syndrome or diabetes mellitus is becoming a more frequent risk factor. (Llovet et al., 2022) . Hepatocellular Carcinoma (HCC) is a formidable global health challenge, representing one of the most prevalent primary liver

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malignancies with increasing incidence rates worldwide. While the therapeutic landscape for HCC encompasses a wide array of interventions, Living Donor Liver Transplantation (LDLT) has emerged as a crucial and innovative approach for the management of advanced HCC. This introduction provides a concise overview of HCC and LDLT, touching upon their respective clinical, epidemiological, and therapeutic dimensions. It sets the stage for a comprehensive exploration of how LDLT has redefined the paradigms of treatment for HCC and contributed to enhanced patient outcomes. The interplay between these two entities in the context of HCC management underscores the significance of a holistic approach that considers both the oncological and surgical aspects of this complex disease. This review seeks to shed light on the multifaceted landscape of HCC and LDLT, offering insights into their interdependence, potential synergies, and ongoing advancements in the field.

Around the world, live donor liver transplantation (LDLT) is an important source of organs for patients with liver disease. Hepatocellular carcinoma (HCC) occurring in the setting of cirrhosis is a common indication for transplantation. Liver transplantation (LT) has been accepted worldwide as the most effective treatment modality for patients with HCC. Cholangiocarcinoma, hemangioendothelioma, and hepatoblastoma are much rarer indications for transplantation. (Brown, 2009). Liver transplantation (LT) has been accepted worldwide as the most effective treatment modality for patients with

HCC and Liver transplantation (LT) represents the gold-standard therapy to cure well-selected patients with hepatocellular cancer (HCC) (Lai, 2022), for selection criteria transplant centers uses MILAN criteria. (Lee, 2016). Milan criteria are commonly used and have been adopted by the United Network of Organ Sharing. Identifies subgroup of patient with primary or secondary liver malignancy who may benefit most from liver transplantation.(Tumor size should be at least 2 cm. Maximum diameter of tumor is 5 cm if single, or no more than three liver tumors with maximum size of 3 cm.) (Lauren & Alexander, 2015). The Milan criteria are used to identify patients with HCC in whom the tumor burden is small enough to allow good outcome after liver transplantation. The aim of this paper is to measure prevalence of HCC among LDLT Recipients in Georgia and clarify its effect on outcome.

Method

To answer the research, question the study used cross-sectional study design with a purposive sampling. Information on interested variables were obtained from the medical documentations of patient's medical history in Batumi Referral Hospital. Study subjects were adult liver recipients who had liver transplantation at our center (Batumi, Referral Hospital) since 2014 including August of 2023, totally 84 recipients Median age 49.5 year. For finding association between histopathological confirmed HCC diagnose and outcome we have used SPSS statistical Analyze.

Results and Discussion

To answer the question of this paper we used cross sectional study design among 84 Living Donor Liver Transplant Recipients. After collecting data analyzing patient's medical cards, we have found that out of 84 recipients 12 (11%) had a Hepatocellular Carcinoma, out of these 8 (6.7%) recipients dead and 4 (3.3%) is alive.

Table 1. Chi-Square tests

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	5.447[a]	1	.020		
Continuity Correction[b]	4.032	1	.045		
Likelihood Ratio	5.257	1	.022		
Fisher's Exact Test				.026	.024
Linear-by-Linear Association	5.376	1	.020		
N of Valid Cases	76				

a. 1 cells (25.0%) have expected count less than 5. The minimum expected count is 4.42.

b. Computed only for a 2x2 table

Analyzing data in chi-square test show that there is significantly tight association between HCC and outcome (p = .02) the discussion about Hepatocellular Carcinoma (HCC) and Living Donor Liver Transplantation (LDLT) encompasses various aspects of these two interconnected subjects. HCC, as one of the most prevalent primary liver malignancies, poses a significant clinical challenge, while LDLT has emerged as a vital approach in the management of advanced HCC. HCC, as a one of the most prevalent primary liver malignancies, poses a significant clinical challenge such as: Diagnostic Challenges, Treatment Options, and Selection Criteria for LDLT in HCC.

Analyzing data in chi-square test show that there is significantly tight association between HCC and outcome (p = .02) the discussion about Hepatocellular Carcinoma (HCC) and Living Donor Liver Transplantation (LDLT) encompasses various aspects of these two interconnected subjects. HCC, as one of the most prevalent primary liver malignancies, poses a significant clinical challenge, while LDLT has emerged as a vital approach in the management of advanced HCC. HCC, as a one of the most prevalent primary liver malignancies, poses a significant clinical challenge such as: Diagnostic Challenges, Treatment Options, and Selection Criteria for LDLT in HCC. The discussion about HCC and LDLT encompasses various aspects such es; Diagnostic Challenges, Treatment Options, Selection Criteria.

Conclusion

Present paper aimed to is to measure prevalence of HCC among LDLT Recipients in Georgia and clarify its effect on outcome and resalts shows significant association between HCC status and outcome.

Recommendations

When it comes think about recommendations for Hepatocellular carcinoma and Living Donor Liver Transplantation, several conditions need to be ensured for the best outcome, such as;

- ✓ Early Diagnosis and Surveillance
- ✓ Multidisciplinary stuff
- ✓ Patient selection criteria
- ✓ Minimizing waiting time
- ✓ Immunosuppression and recurrence prevention
- ✓ And most important part for country which has started innovative service to give their citizens chance to prolong life is to share and collaborate with other transplant centers and research institutions to improve outcomes.

Scientific Ethics Declaration

The authors declare that the scientific ethical and legal responsibility of this article published in EPHELS journal belongs to the authors.

Acknowledgements or Notes

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References

Azzam, A. Z. (2015). Liver transplantation as a management of hepatocellular carcinoma. *World Journal of Hepatology, 7*(10), 1347.

Brown, J. R. S. (2009). Live donors in liver transplantation. *Gastroenterology, 134*(6), 1802-1813.
Lai, Q., Viveiros, A., Vitale, A.,…& Lerut, J. P. (2022). Prognostic factors for 10-year survival in patients with hepatocellular cancer receiving liver transplantation. *Frontiers in Oncology, 12,* 877107.
Lauren, F., & Alexander, R. S. (2015). *Textbook of gastrointestinal radiology*.(Vol. 2, 4 th ed.).
Lee, H. W., & Suh, K. S. (2016). Expansion of the criteria for living donor liver transplantation for hepatocellular carcinoma. *Current Opinion in Organ Transplantation, 21*(2)*,* 231-237.
Llovet, J. M., Kelley, R. K., Villanueva, A.,…& Finn, R. S. (2022). Hepatocellular carcinoma. *Nature reviews. Disease Primers,7*(1), 6.

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The Effect of Nitrate Supplementation on Sportive Performance

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Abstract: Beetroot juice is typically used as a source of dietary nitrate supplementation, which has been hailed as a potential new ergogenic aid for athletic and exercise performance. A 0.6% performance boost is now deemed adequate to make a difference due to the rise in competitive equality in top-level sport especially. The aim of this study is to examine the effects of nitrate supplementation on sports performance and to reveal the mechanisms of action. For that, information from the publications evaluated throughout the scope of SCI, SCI-Expanded, and ESCI were collected by scanning the literature in the SportDiscus, EMBASE, PubMed, and Google Scholar databases over a 10-year period. According to literature, it has been stated that nitrate supplementation provides vasodilation, triggers mitochondrial biogenesis, glucose uptake, and calcium transport from the sarcoplasmic reticulum. Nitrate supplementation has been demonstrated to have physiological effects that may be advantageous for improving exercise performance, at least in recreationally active or sub-elite athletes.

Keywords: Beetroot, Nitric oxide, Phytochemicals

Introduction

Numerous vascular and cellular processes, such as cellular respiration, vasodilation, and angiogenesis, depend on the signaling molecule nitric oxide (NO). Through both endogenous and exogenous mechanisms, dietary nitrate (NO_3^-) consumption causes the production of NO (Clements et al., 2014). Nitric oxide, which is known to play a number of significant roles in vascular and metabolic control, can be produced from nitrite under situations of insufficient oxygen accessibility. In addition to this, plasma nitrite levels is raised by dietary nitrate intake (Jones, 2014). Nitric oxide is a substance produced from L-arginine by the enzyme nitric oxide synthetase, and since its half-life is very short, it turns into inactive nitrite (NO_2^-) and nitrate (NO_3^-). In addition to its production in the body, it can also be taken externally through diet (Maughan, 2018). Some veggies contain an active substance called NO_3^- that may have ergogenic and health-improving benefits. Therefore, NO_3^- appears as an intriguing substance from the perspectives of both sports as an ergogenic aid and as a possibly affordable strategy for lowering the likelihood of cardiovascular events (Clements et al., 2014). All the same, for the past ten years, dietary nitrate (NO_3^-) supplementation has been studied for its potential ergogenic effects (Senefeld et al., 2020). This review's goal is to clarify the mechanisms by which nitrate supplementation improves athletic performance. Promoting the nitrate-nitrite-NO pathway's bioavailable of NO may have an impact on how well muscles work during exercise. By controlling blood flow, contractility, glucose and calcium homeostasis, as well as mitochondrial respiration and biogenesis, NO plays a role in the controlling of the functioning of skeletal muscles.

Shortly, nitrate supplementation affects sportive performance in a number of ways: provides vasodilation, triggers mitochondrial biogenesis, glucose uptake, and calcium transport from the sarcoplasmic reticulum (Jones, 2014). All these pathways will be examined from a biochemical perspective in this review.

Vasodilatation Effect

Nitrate needs some enzymatic processes to turn into nitric oxide. Nitrate groups interact with enzymes and sulfhydryl groups that remove nitrate groups. By this way, nitric oxide converts Guanylyl Cyclase (GC) to 3'-5'-cyclic guanosine monophosphate (cGMP). cGMP reduces the strength of cardiac contractions in cardiac cells by stimulating the ion pumps that enable Ca^{2+} to be removed from the cytoplasm and by reducing the Ca^{2+} sensitivity of the contractile apparatus. Increasing intracellular cGMP concentration inhibits the entry of Ca+2 into the cell, thus decreasing the intracellular Ca^{+2} concentration. Veins expand. Additionally, nitric oxide opens K^+ channels, causing hyperpolarization and relaxation. Finally, NO can activate myosin light chain phosphatase, an enzyme that dephosphorylates myosin light chains and causes relaxation, by stimulating a cGMP-dependent protein kinase (Gormus & Ozmen, 2005). Enhancing the NO_3^- / NO_2^- / NO pathway may have an impact on how well muscles work during exercise. NO's function in skeletal muscle contractility can modify that muscle's activity (Stamler & Meissner, 2001).

Mitochondrial Biogenesis

The genomic process that produces new mitochondria, known as mitochondrial biogenesis, is essential for preserving an adequate amount of mitochondria and is the basis for adaptations that are triggered by external stimuli like exercising (Holloszy,1967). Nitrate, is a well-known mediator of mitochondrial activity that promotes the synthesis of cyclic guanosine monophosphate (cGMP) and soluble guanylate cyclase (sGC) to promote mitochondrial biogenesis (Nisoli et al., 2005).

The relationships between nitrate, nitrite, NO, and the mitochondria are many. The effect of NO that has been studied the most may be its binding to cytochrome coxidase (COX), the electron transport system's (ETS) terminal electron acceptor, which partially inhibits the respiration of mitochondria (Brown & Cooper, 1994). Dynamic and oxygen-regulated, this binding may also be involved in tissue oxygen gradient regulation and reactive oxygen species (ROS) signaling regulation. Furthermore, NO promotes mitochondrial biogenesis via a process that is dependent on cGMP. Because proteins in the ETS can convert nitrite to NO, the effects of nitrite may resemble those of NO. Lastly, nitrite may signal to control tissue protein expression and activation without the need for NO synthesis (Nisoli et al., 2003).

The traditional method of measuring mitochondrial oxidative phosphorylation efficiency is the P/O ratio, which is the quantity of oxygen used for every ATP generated. After receiving nitrate supplementation, muscle tissue mitochondria were extracted and showed improved oxidative phosphorylation efficiency (P/O ratio). There was a correlation between the decrease in oxygen cost during exercise and the enhanced mitochondrial P/O ratio. Nitrate lowered the expression of ATP/ADP translocase, a proton conductance-related protein, mechanistically (Larsen et al., 2011).

Glucose Uptake

Throughout physical activity, glucose is a key fuel source, and during activity bouts, skeletal muscle can absorb glucose up to 50 times more efficiently.Exercise length and intensity are important factors that affect how well skeletal muscle absorbs glucose.The intracellular metabolic state controls the glucose transport triggered by exercise (controlled by AMPK) (Sylow et al., 2007).The protein kinase needed for glucose-repressed transcription is called AMPK (Chen et al., 1999).

The translocation of GLUT4 to the cell surface is enhanced by contraction and exercise, which facilitates the transport of glucose into muscle cells. Though the exact mechanisms by which contraction boosts GLUT4 translocation and muscle glucose uptake remain unclear, it is generally agreed that the signals controlling this process are distinct from those governing the insulin-signaling system.Many mechanisms have been proposed to control contraction-stimulated glucose absorption, with AMP-activated protein kinase (AMPK) being one of the more appealing and thoroughly researched possibilities (Lira et al., 2007). The translocation of GLUT4 and other catabolic activities are increased when AMPK is pharmacologically activated (Lee-Young et al., 2009).

It has been suggested that NO regulates contraction and NO-mediated glucose absorption by acting both upstream and downstream of AMPK. On the other hand, it has been demonstrated that NOS inhibition minimizes glucose absorption without altering AMPK signaling during contractions. In muscles with a higher percentage of glycolytic fibers, NO has a role in controlling glucose absorption during contraction independently of AMPK (Lee-Young et al., 2009). The phosphorylation of NOS by AMPK results in a rise in

NOS activation (Chen et al., 1999). Briefly, nitrate controls the uptake of glucose by muscles in the skeleton during exercise in a manner that is independent of AMPK.

Calcium Transport to Sarcoplasmic Reticulum

Sarcoplasmic reticulum (SR), Ca^{2+} release in isolated skeletal muscle fibers decreases significantly in the later phases of exhaustion (Westerblad et al., 1998). By modifying Ca^{2+} release and absorption by the sarcoplasmic reticulum, NO controls muscle contraction. After nitrate supplementation, the impact of NO on these systems may enhance exercise efficiency (Hoon et al., 2015).

Dihydropyridine receptor (DHPR) and calsequestrin 1 (CASQ1) are two proteins that bind $Ca^{2+.}$ A rise in DHPR would facilitate the release of Ca^{2+} more easily throughout contraction, whereas higher CASQ1 expression and intracellular [Ca^{2+}] probably increase the quantity of releasable Ca^{2+} from the sarcoplasmic reticulum during excitation–contraction coupling (creating extra actin–myosin binding sites) (Hernández et al., 2012). Furthermore, after supplementation, there was a stated rise in metabolic efficiency, which decreased the accumulation of intracellular phosphate ([Pi]) throughout contraction. Considering that raised [Pi] has been shown to reduce Ca^{2+} sensitivity and sarcoplasmic reticulum Ca^{2+} release to sarcoplazma, this may further help to preserve the muscle force found throughout exhaustive exercise (Duke & Steele, 2001). Shortly, since nitrate supplementation increased NO generation, there may have been a decrease in skeletal muscle ATP turnover through a decrease in actomyosin ATPase and/or Ca^{2+}-ATPase activity (Bailey et al., 2010).

Method

In order to gather information from publications that were reviewed within the scope of SCI, SCI-Expanded, and ESCI for this review, the literature in the SportDiscus, EMBASE, PubMed, and Google Scholar databases was scanned over a ten-year period using the keywords "nitrate" AND "sportive performance supplements" AND "nitric oxide." A thorough analysis of the chosen studies was done in an effort to describe how nitrate has ergogenic effects.

Conclusion

According to recent research, NO3− is a helpful ergogenic aid that can be used in a range of sports and exercise scenarios to enhance performance through a variety of processes (Macuh & Knap, 2021). Nitrate supplementation has been reported in studies to promote vasodilation, mitochondrial biogenesis, glucose uptake, and sarcoplasmic reticulum calcium transfer. At least in recreationally active or sub-elite athletes, nitrate supplementation has been shown to produce physiological responses that may be beneficial for enhancing athletic performance.

Recommendations

When given acutely or chronically in the range of 300–1041 mg NO3−, 2-3 hours before to exercise, and mainly in the case of exercise duration of 10–17 min, the effect of NO3− is most noticeable in less-trained individuals (Macuh & Knap, 2021). That is the case with any sports supplements, one must evaluate the risk against the potential benefits. Since nitrate can be consumed in naturally occurring forms like vegetables and beetroot juice, this may reduce the chance of consuming illegal substances and provide additional health advantages linked to higher dietary nitrate intake. In addition to the small number of research findings indicating adverse effects, nitrate supplements appear to be a low risk intervention that could improve performance during exercises (Hoon et al.,2013).

Scientific Ethics Declaration

The author declares that the scientific ethical and legal responsibility of this article published in EPHELS journal belongs to the author.

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References

Bailey, S. J., Fulford, J., Vanhatalo, A., Winyard, P. G., Blackwell, J. R., DiMenna, F. J., ... & Jones, A. M. (2010). Dietary nitrate supplementation enhances muscle contractile efficiency during knee-extensor exercise in humans. *Journal of Applied Physiology*, *109*(1), 135-148.

Brown, G. C., & Cooper, C. E. (1994). Nanomolar concentrations of nitric oxide reversibly inhibit synaptosomal respiration by competing with oxygen at cytochrome oxidase. *FEBS Letters*, *356*(2-3), 295–298.

Chen, Z. P., Mitchelhill, K. I., Michell, B. J., Stapleton, D., Rodriguez-Crespo, I., Witters, L. A., ... & Kemp, B. E. (1999). AMP-activated protein kinase phosphorylation of endothelial NO synthase. *FEBS letters*, *443*(3), 285-289.

Clements, W. T., Lee, S. R., & Bloomer, R. J. (2014). Nitrate ingestion: a review of the health and physical performance effects. *Nutrients*, 6(11), 5224-5264.

Duke, A. M., & Steele, D. S. (2001). Mechanisms of reduced SR Ca2+ release induced by inorganic phosphate in rat skeletal muscle fibers. *American Journal of Physiology-Cell Physiology*, *281*(2), 418-29.

Gormus, U., & Ozmen, D. (2005). cGMP ve klinik önemi. *Türkiye Klinikleri Tıp Bilimleri Dergisi*, *25*(5), 678-87.

Hernández, A., Schiffer, T. A., Ivarsson, N., Cheng, A. J., Bruton, J. D., Lundberg, J. O., ... & Westerblad, H. (2012). Dietary nitrate increases tetanic [Ca2+] and contractile force in mouse fast-twitch muscle. *The Journal of Physiology*, *590*(15), 3575-3583.

Holloszy, J. O. (1967). Biochemical adaptations in muscle: effects of exercise on mitochondrial oxygen uptake and respiratory enzyme activity in skeletal muscle. *Journal of Biological Chemistry*, *242*(9), 2278-2282.

Hoon, M. W., Johnson, N. A., Chapman, P. G., & Burke, L. M. (2013). The effect of nitrate supplementation on exercise performance in healthy individuals: A systematic review and meta-analysis. *International Journal of Sport Nutrition and Exercise Metabolism*, *23*(5), 522-532.

Hoon, M. W., Fornusek, C., Chapman, P. G., & Johnson, N. A. (2015). The effect of nitrate supplementation on muscle contraction in healthy adults. *European Journal of Sport Science*, *15*(8), 712–719.

Jones, A. M. (2014). Dietary nitrate supplementation and exercise performance. *Sports Medicine, 44* (1), 35–45.

Larsen, F. J., Schiffer, T. A., Borniquel, S., Sahlin, K., Ekblom, B., Lundberg, J. O., & Weitzberg, E. (2011). Dietary inorganic nitrate improves mitochondrial efficiency in humans. *Cell Metabolism*, *13*(2), 149-159.

Lee-Young, R. S., Griffee, S. R., Lynes, S. E., Bracy, D. P., Ayala, J. E., McGuinness, O. P., & Wasserman, D. H. (2009). Skeletal muscle AMP-activated protein kinase is essential for the metabolic response to exercise in vivo. *Journal of Biological Chemistry*, *284*(36), 23925-23934.

Lira, V. A., Soltow, Q. A., Long, J. H., Betters, J. L., Sellman, J. E., & Criswell, D. S. (2007). Nitric oxide increases GLUT4 expression and regulates AMPK signaling in skeletal muscle. *American Journal of Physiology-Endocrinology and Metabolism*, *293*(4), 1062-68.

Macuh, M., & Knap, B.(2021). Effects of nitrate supplementation on exercise performance in humans: A narrative review. *Nutrients,13*(9), 3183.

Maughan, R.J. (2018). IOC medical and scientific commission reviews its position on the use of dietary supplements by elite athletes. *British Journal of Sports Medicine*, *52*(7),418-419.

Nisoli, E., Clementi, E., Paolucci, C., Cozzi, V., Tonello, C., Sciorati, C., Bracale, R., Valerio, A., Francolini, M., Moncada, S., & Carruba, M. O. (2003). Mitochondrial biogenesis in mammals: the role of endogenous nitric oxide. *Science*, *299*(5608), 896–899.

Nisoli, E., Tonello, C., Cardile, A., Cozzi, V., Bracale, R., Tedesco, L., ... & Carruba, M. O. (2005). Calorie restriction promotes mitochondrial biogenesis by inducing the expression of eNOS. *Science*, *310*(5746), 314-317.

Senefeld, J. W., Wiggins, C. C., Regimbal, R. J., Dominelli, P. B., Baker, S. E., & Joyner, M. J. (2020). Ergogenic effect of nitrate supplementation: A systematic review and meta-analysis. *Medicine and Science in Sports and Exercise*, *52*(10), 2250–2261.

Stamler, J. S., & Meissner, G. (2001). Physiology of nitric oxide in skeletal muscle. *Physiological Reviews*, *81*(1), 209–237.

Sylow, L., Kleinert, M., Richter, E. A., & Jensen, T. E. (2017). Exercise-stimulated glucose uptake - regulation and implications for glycaemic control. Nature reviews. *Endocrinology*, *13*(3), 133–148.

Westerblad, H., Allen, D. G., Bruton, J. D., Andrade, F. H., & Lännergren, J. (1998). Mechanisms underlying the reduction of isometric force in skeletal muscle fatigue. *Acta Physiologica Scandinavica*, *162*(3), 253-260.

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Investigation of Potential Effects of Aronia Melonacarpa Supplementation on Sportive Performance

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Abstract: Nowadays, people's interest in more natural resources and aromatic plants has started to increase in terms of natural and healthy nutrition. Recently, the demand for medicinal plants rich in polyphenols with antimicrobial and antioxidant properties has increased. Aronia (Aronia melanocarpa) has recently become one of the most preferred fruits due to its high antioxidant activity and rich polyphenol content. The aim of this study was to investigate the potential effects of Aronia supplementation on athletes and its mechanisms of action from a specific point of view. For this purpose, a literature review was conducted and information was compiled with current studies by searching through aronia supplementation and sportive performance. It is seen that Aronia melanocarpan is used in many aspects. It has been evaluated in the treatment of many diseases, including colds and stomach diseases, as well as intestines, gall bladder and liver. Since it increases the level of good cholesterol, it has also been used to combat heart disease and cardiovascular problems. As a result of the literature researches, it can be said that aronia melanocarpan is used for therapeutic purposes in many diseases, but the studies in terms of sportive performance are limited. More studies are needed to reveal the effects of this supplement on sportive performance.

Keywords: Supplement, Sport, performance, Antioxidant

Introduction

Aronia melanocarpa spread to Russia under the leadership of Germany in the early 1900s and started production. This remarkable plant is a shrubby species belonging to the Rosaceae family and can reach a height of 2 to 3 metres. Flowering in May-June, Aronia bears fruits that are shiny black in colour when ripe, measuring 6 to 13 mm in size and weighing 0.5 to 2 grams (Chrubasik et al., 2010). In different parts of Europe, Aronia melanocarpa fruit is found in various uses in the form of fruit syrup, jam, marmalade, tea, juice and extracts. This fruit is commonly known as "Chokeberry" and is divided into two recognised species, Aronia melanocarpa (black chokeberry) and Aronia arbutifolia (red chokeberry) (Oszmiański & Lachowicz, 2016). Aronia fruit stands out as a functional food due to its rich chemical content and high antioxidant activity. It is attracting more and more attention worldwide and its health benefits are being investigated.

In particular, its significant antioxidant activity plays an effective role in the treatment of chronic and degenerative diseases (Konić-Ristić et al., 2011). The positive effects of anthocyanins on health have also focused research on the content of Aronia melanocarpa fruit. This fruit offers a wide range of protective and beneficial effects based on its antioxidant and biological activities (Tolic et al., 2017). In this context, the potential of Aronia melanocarpa in the field of health and nutrition is attracting more and more interest worldwide. This research focussed on the use of the natural food supplement in the context of exercise.

Method

This study is a study obtained by summarizing many studies using the compilation method. A compilation is expressed as articles created by bringing together published studies on a particular subject. The purpose of the reviews is to present the information about that subject to the reader as a whole (Karaçam, 2013). For this purpose, literature review SCI, SSCI, SCI-Expanded and sports basic field articles accessed from SportDiscus, Google Scholar, PubMed and EMBASE databases were examined and the information was compiled and presented with a holistic perspective.

Results and Discussion

Antioxidant Content and Application Areas

Aronia melanocarpa stands out as a food source enriched with various valuable compounds such as anthocyanin, proanthocyanidin, flavonol, flavanol and phenolic acids (Kokotkiewicz et al. 2010). This special fruit has a number of positive effects on health due to its notorius antioxidant profile. Aronia fruits are also used as herbal medicine in Russia and Eastern European countries, not only for their nutritional properties, but also for their antihypertensive and anti-atherosclerotic properties (Jurikova et al., 2017).

The various properties of this fruit, which is important for health, have been elucidated by many studies. Thanks to its antioxidant properties, many studies have focused on the anti-inflammatory, antibacterial, anticancer, antimutagenic and antidiabetic activities of Aronia melanocarpa (Naruszewicz et al., 2007). In addition, research on obesity has shed light on the therapeutic potential of Aronia. This special fruit also stands out as a potential aid in the prevention and treatment of autoimmune and cardiovascular diseases. The health benefits of Aronia melanocarpa are evaluated in a wide spectrum and the powerful biological effects of this fruit have aroused a growing interest in the medical world.

Furthermore, these beneficial properties of Aronia can be integrated with traditional medical practices and become part of a holistic understanding of health supported by natural and herbal solutions as well as modern medicine. In conclusion, the nutritional value of Aronia melanocarpa and its positive effects on health emphasise the versatile use of this fruit and its importance in the world of health. Further research and discoveries in the future will allow us to better understand the potential of Aronia and more effectively understand the role of this valuable fruit in the field of health.

Aronia melanocarpa is a fruit that attracts attention with its rich components such as anthocyanin, proanthocyanidin, flavonol, flavanol and phenolic acid. In particular, research shows that this fruit has a number of positive effects on health. Aronia fruit juice has hepatoprotective (liver protective), hypolipidemic (lowering lipid levels) and hypoglycaemic (lowering blood sugar) effects. Antiaggregant, anti-inflammatory, vasoactive, vasoprotective and hypotensive activities of aronia extracts are also indicated. Various studies indicate that the rich content of this fruit in polyphenols, especially anthocyanins, is the main reason for these favourable effects, being associated with its antiradical activity in vitro and in vivo. However, the wide range of constituents contained in aronia fruit may not only be limited to antioxidant effects, but may also act on different mechanisms on the organism (Bell et al., 2006; Valcheva et al., 2007; Valcheva-Kuzmanova et al., 2004; Maas et al., 2016).

These positive health effects of this fruit suggest that it may play an important role in the prevention of type 2 diabetes, cardiovascular diseases and their potential complications. Common risk factors for type 2 diabetes and cardiovascular diseases include impaired glucose tolerance (prediabetic state) and dyslipidaemia. These conditions, taken together, can lead to metabolic syndrome. Research on the potential of the bioactive components contained in aronia fruit to reduce the possible effects of this metabolic syndrome suggests that this fruit may have an important role in health. Therefore, aronia fruit and its derivatives are recommended by nutritionists and researchers as part of a healthy lifestyle, and studies on it have contributed to a better understanding of its contributions to health (Bell et al., 2006; Valcheva et al., 2007; Valcheva-Kuzmanova et al., 2004; Maas et al., 2016).

In a study examining the effects of aronia fruit juice, multiple pathways were tested on insulin signalling to focus on reactions related to insulin resistance and proved to reduce the risk factor. In a separate study, aronia extract was observed to reduce fasting blood glucose levels in individuals with type 2 diabetes (Valcheva et al., 2007). In another study, the effects of aronia fruit juice were examined in diabetic and healthy rats. This study revealed a significant decrease in abnormalities in the diabetic group. Cardioprotective studies have shown a

significant decrease in total cholesterol and low-density cholesterol levels in patients with metabolic syndrome (Bell et al., 2006).

When the effects of aronia extract on lipid parameters were investigated, it was observed that total cholesterol level decreased, triglyceride levels decreased and HDL2 cholesterol increased. In another study, it was reported that aronia fruit juice caused a concentration-dependent decrease in superoxide production only in individuals with cardiovascular risk, while no effect was observed in the control group (Bell et al., 2006). The levels of antioxidants increased with the administration of aronia supplementation in athletes and effectively strengthened the glutathione defence system by increasing GSH availability and GPx activity after exercise and 30 minutes after exercise (Chung et al., 2023). Currently, studies on the use of this supplement in sportive fields are increasing in the literature.

Conclusion

Research has clearly demonstrated that aronia fruit juice and extract exhibit positive effects on health. These positive effects on a wide range of health parameters, from insulin resistance to cardiovascular risk factors, emphasise the nutritional and therapeutic potential of aronia. There is increasing scientific evidence supporting the positive effects of the bioactive components contained in this fruit, especially anthocyanins, on metabolism. These positive contributions of aronia to health lead it to be considered as a potential natural supplement in the prevention and treatment of many health problems.

Recommendations

Especially its positive effects on insulin resistance and risk factors of cardiovascular diseases are based on the strong antioxidant and anti-inflammatory properties of this fruit. In conclusion, the positive effects of the rich bioactive components in aronia fruit on health have been supported by scientific research and emphasised that this fruit can play an important role in the world of health. The potential of this natural resource will be further understood by future research and evaluated as an effective support in healthy lifestyle practices.

Scientific Ethics Declaration

The authors declare that the scientific ethical and legal responsibility of this article published in EPHELS journal belongs to the authors.

Acknowledgements or Notes

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References

Bekinschtein, P., Cammarota, M., Izquierdo, I., & Medina, J. H. (2008). Reviews: BDNF and memory formation and storage. *The Neuroscientist*, *14*(2), 147-156.

Bell, D. R., & Gochenaur, K. (2006). Direct vasoactive and vasoprotective properties of anthocyanin-rich extracts. *Journal of Applied Physiology*, *100*(4), 1164-1170.

Chrubasik, C., Li, G., & Chrubasik, S. (2010). The clinical effectiveness of chokeberry: a systematic review. *Phytotherapy Research*, *24*(8), 1107-1114.

Chung, J. W., Kim, J. E., Nam, Y. E., Kim, W. S., Lee, I., Yim, S. V., & Kwon, O. (2023). Eight-week supplementation of Aronia berry extract promoted the glutathione defence system against acute aerobic exercise-induced oxidative load immediately and 30 min post-exercise in healthy adults: a double-blind, randomised controlled trial. *Journal of Human Nutrition and Dietetics*. https://doi.org/10.1111/jhn.13150

Jurikova, T., Mlcek, J., Skrovankova, S., Sumczynski, D., Sochor, J., Hlavacova, I., ... & Orsavova, J. (2017). Fruits of black chokeberry Aronia melanocarpa in the prevention of chronic diseases. *Molecules*, *22*(6), 944.

Kokotkiewicz, A., Jaremicz, Z., & Luczkiewicz, M. (2010). Aronia plants: a review of traditional use, biological activities, and perspectives for modern medicine. *Journal of Medicinal Food*, *13*(2), 255-269.

Konić-Ristić, A., Šavikin, K., Zdunić, G., Janković, T., Juranic, Z., Menković, N., & Stanković, I. (2011). Biological activity and chemical composition of different berry juices. *Food Chemistry*, *125*(4), 1412-1417.

Kowiański, P., Lietzau, G., Czuba, E., Waśkow, M., Steliga, A., & Moryś, J. (2018). BDNF: a key factor with multipotent impact on brain signaling and synaptic plasticity. *Cellular and Molecular Neurobiology*, *38*, 579-593.

Maass, A., Düzel, S., Brigadski, T., Goerke, M., Becke, A., Sobieray, U., ... & Düzel, E. (2016). Relationships of peripheral IGF-1, VEGF and BDNF levels to exercise-related changes in memory, hippocampal perfusion and volumes in older adults. *Neuroimage*, *131*, 142-154.

Naruszewicz, M., Łaniewska, I., Millo, B., & Dłużniewski, M. (2007). Combination therapy of statin with flavonoids rich extract from chokeberry fruits enhanced reduction in cardiovascular risk markers in patients after myocardial infraction (MI). ??*Atherosclerosis*, *194*(2), 179-184.

Oszmiański, J., & Lachowicz, S. (2016). Effect of the production of dried fruits and juice from chokeberry (Aronia melanocarpa L.) on the content and antioxidative activity of bioactive compounds. *Molecules*, *21*(8), 1098.

Tolic, M. T., Krbavcic, I. P., Vujevic, P., Milinovic, B., Jurcevic, I. L., & Vahcic, N. (2017). Effects of weather conditions on phenolic content and antioxidant capacity in juice of chokeberries (Aronia melanocarpa L.). *Polish Journal of Food and Nutrition Sciences*, *67*(1), 67-74

Valcheva-Kuzmanova, S., Borisova, P., Galunska, B., Krasnaliev, I., & Belcheva, A. (2004). Hepatoprotective effect of the natural fruit juice from Aronia melanocarpa on carbon tetrachloride-induced acute liver damage in rats. *Experimental and Toxicologic Pathology*, *56*(3), 195-201.

Valcheva-Kuzmanova, S., Kuzmanov, K., Tancheva, S., & Belcheva, A. (2007). Hypoglycemic and hypolipidemic effects of Aronia melanocarpa fruit juice in streptozotocin-induced diabetic rats. *Methods and Findings in Experimental and Clinical Pharmacology*, *29*(2), 101-106.

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Assessment of Occupational Risks Associated with Chemical Reagents in the Hemobiology Laboratory of an Eastern Algerian University Hospital Center

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Abstract: The variety and diversity of chemicals handled in hospital laboratories make it imperative to conduct periodic risk assessments for enhanced protection of medical personnel and the implementation of an appropriate prevention policy. It is within this context that our study was conducted in the hemobiology laboratories of an eastern Algerian university hospital center. We employed a semi-quantitative assessment method tailored to the specificities of hospitals. An inventory of chemical agents was conducted using data collection sheets detailing the product names, quantities, and frequencies of use. Relying on information from the Safety Data Sheet (SDS) and associated pictograms.We characterized the hazards posed by these chemicals and prioritized their associated risks. The hemobiology laboratory staff use 49 chemical reagents, with 28% of them considered hazardous, demanding appropriate safety precautions. The study highlighted the significance of systemic and local cutaneous effects, such as sensitivity (94.6%). Ocular risk, with severe injuries, was caused by 33.3% of the reagents. Seventeen reagents could lead to respiratory systemic effects. Ten reagents posed a carcinogenic, mutagenic, and reprotoxic (CMR) risk, such as Chloroform, Formaldehyde, ADVIA Perox 1, ADVIA Perox 2, ADVIA Perox 3. This evaluation has highlighted the multiple health risks faced by the personnel in the hemobiology laboratory. It is imperative to establish a comprehensive strategy for managing occupational risks to ensure the long-term protection of the workers.

Keywords: Chemical reagents, Chemical risks, Hemobiology laboratory, Risk assessment.

Introduction

Hospital laboratories, especially those specialized in hemobiology, expose their personnel to significant occupational risks due to the use of potentially hazardous chemicals. This arises from the very nature of laboratory activities and the diversity of chemicals handled. To date, the exposure of hospital staff to chemical agents remains poorly understood. (Berrubé and al., 2013; Baurès , 2016). The assessment of chemical risk is often less systematic and less prioritized.

However, the handled chemical reagents possess numerous toxic, corrosive, and flammable properties, which can have detrimental consequences on the health of personnel and the environment. Thus, the significant exposure of handlers to chemical reagents and the inefficacy of preventive measures underscore the importance of managing this risk to safeguard their health and ensure a higher quality of analyses. Ultimately, this contributes to the protection of patients' health (Dumas & Persoons, 2004; Vincentet al., 2005).

To ensure the safety of personnel, it is imperative to conduct periodic risk assessments, given the diversity of chemicals used. Moreover, substances classified as Carcinogenic, Mutagenic, and Reprotoxic (CMR) are subject to stringent regulations regarding their handling and the use of appropriate protective equipment. Consequently, their identification is a crucial preliminary step to enhance information and personnel protection (Dumas & Persoons, 2004; INRS, 2023a, 2023c). These assessments are essential for establishing an appropriate prevention policy and enhancing the protection of medical personnel in hospital laboratories (INRS, 2023b). In this context, a semi-quantitative methodology for the assessment of chemical risk was conducted in the hemobiology laboratories of a university hospital center in eastern Algeria.

Materials and Method

This is a cross-sectional descriptive study conducted at the hemobiology laboratory of an Algerian university hospital center. For the assessment of chemical risk, we opted for a semi-quantitative evaluation method adapted to the specificities of hospitals. The data for our study were collected from various sources to provide a comprehensive approach to the evaluation of chemical risks.

Data Inventory

We utilized a census form to systematically record specific information regarding the overall organization of hemobiology laboratories and units, equipment, medical, paramedical, and technical-administrative personnel, as well as tasks performed and the frequency of their execution. An inventory of chemical reagents was conducted using data collection forms that included product names, quantities, physical and chemical characteristics, toxic properties, usage precautions, handling procedures, storage measures, and frequency of use.

The commercial name of the product and the supplier's name were used to access the chemical product's Safety Data Sheet (SDS). The information provided in an SDS is categorized based on various criteria, including the nature of the preparation (hazardous or non-hazardous), the concentration of the chemical agent in the preparation, the presence of an occupational exposure limit (OEL), and potential risks to health and/or the environment. (Berrubé et al., 2013).

Chemical Risk Prioritization

Classes of Hazard

Based on the information provided in the SDS and associated pictograms, we assessed the hazards posed by the chemical products and established a hierarchy of associated risks. Health effects are categorized into three levels of hazard: Level 1 (slightly or moderately hazardous), Level 2 (hazardous), and Level 3 (highly hazardous). The risks associated with chemical products are linked to their toxicological, physico-chemical, and eco-toxicological properties. However, the method we employed focuses solely on toxicological hazards, which are further categorized into 9 hazard classes based on their effects on health. The prioritization of hazards is limited to the risk phrases (R phrases present in the SDS).

Exposure Analysis

To analyze personnel exposure to chemical hazards, we carefully specified the frequency of handling, the quantities manipulated, and the effectiveness of protective devices. Based on the hazard levels identified by the R risk phrases, it is possible to calculate the Hazard Index (HI) using the following formula:

IH = 10^ (hazard level)

An Exposure Index (EI) defines the potential exposure criterion and varies from 0.1 to 1. It is calculated using the following formula:

EI = 0,1 x « frequency » x « quantity of product » (Persoons and al., 2008)

We also calculated the risk index using the following formula, which helps prioritize preventive actions based on the level of risk.

Risk Index = Hazard Index x Exposure Index x Protection Index (CNRACL, 2007)

The various risk scenarios are documented in Table 1.

Table 1. Correspondence between RI, risk level, and the type of situations.

Risk Index	Risk Level	Situation
RI < 4	Low	Non-priority
4 ≤ RI ≤ 40	Moderate	Intermédiaires. May require further assessment.
RI ≥ 40	High	Priority

Results and Discussion

After compiling an inventory of 49 chemical products, specifically identifying them by their commercial names, we then proceeded to assess the associated hazard levels of these products and calculate the corresponding risk indices. In our study, we found that 28% of the chemical products were hazardous, necessitating the implementation of appropriate safety measures. This categorization classifies them into a high-risk category, requiring special attention. These products included dyes used in cytology such as May Grunwald Giemsa, certain fixatives like methanol and formaldehyde, and solvents such as chloroform and phenol.

The data analysis reveals that 27% of the reagents pose a low risk of causing systemic skin reactions, as their risk index is below 4, categorizing them as acceptable risk. Additionally, 40.6% of the reagents display a skin irritation risk index below 4, indicating a low risk, also placing them in the acceptable risk category. Furthermore, 27% of the reagents obtain a risk index below 4 for skin burns upon exposure, signifying a low risk in this scenario. Examples of these types of chemical reagents are listed in Table 2.

Table 2. Examples of chemical products with skin risk, accompanied by their hazard level and risk index.

Effect Type	Chemical Products	Hazard Level	Risk Index	Risk Level
Skin Irritation	Methylene Blue	1	0.06	
	Hematein	1	0.06	
	Chloroform	1	0.02	27 %
	Brilliant Cresyl Blue	1	0.04	Low risk
	ADVIA perox1, 2 et 3	1	0.04	
Skin Burn	Formaldehyde	2	0.6	
	Hydrochlorique Acide	2	0.4	40.6 %
	Aqueous Phenol	2	0.2	Low risk
	CELL Clean	2	0.2	
Systemic Skin	MAY GRUNWALD solution	2	0.6	
Toxicity	GIEMSA solution	2	0.6	
	Formaldehyde	2	0.6	27 %
	Xylene	1	0.02	Low risk
	Methanol	2	0.6	

The analysis of ocular risks reveals a diversity among the examined chemicals. Approximately 12.5% of these products pose a minimal risk of ocular irritation, considered acceptable. In contrast, 18.8% of them exhibit a moderate risk of ocular irritation, requiring further assessment. Only 2% display a high risk in terms of ocular irritation. Finally, 29% of the chemicals receive a high-risk index for severe ocular injuries, placing them in a category of unacceptable risk. Examples of these types of chemical reagents are listed in Table 3.

Table 3. Examples of chemicals with ocular risk, along with their hazard level and risk index.

Effect type	Chemical products	Hazard level	Risk index	Risk level
Eye irritation	MAY GRUNWALD solution	1	6	
	GIEMSA solution	1	6	- 12.5 % Low risk
	Methylene Blue	1	6	- 18.8% Moderate
	Brilliant Cresyl Blue	1	4	risk
	CELL Clean / CELL Pack	3/1	200/2	2 % High risk
	ADVIA perox1, 2 et 3	1	0.04	
Severe eye injuries	Formaldehyde	3	600	
	Hydrochloric acid	3	400	
	Aqueous Phenol	3	200	29 % High risk
	Chloroform	3	200	
	Potassium ferrocyanide	3	600	

Regarding respiratory risks, it should be noted that: 6% of the chemicals have a moderate risk index for systemic respiratory effects, placing them in an intermediate category that requires a comprehensive assessment of chemical risks. In contrast, 21% of the chemicals have a high-risk index for systemic respiratory effects, categorizing them as a priority group associated with an unacceptable risk. Finally, 8% of the chemicals pose a high risk of local respiratory effects, placing them in a priority category associated with an unacceptable risk. Examples of these types of chemical reagents are listed in Table 4.

Table 4. Examples of chemicals with respiratory risks, along with their hazard level and risk index.

Effect type	Chemical Products	Niveau de danger	Indice de risque	Niveau de risque
Systemic respiratory effect	MAY GRUNWALD solution	2	60	- 6%
	GIEMSA solution	2	60	Moderate
	Formaldehyde	2	60	risk
	Xylene	2	20	- 21 % High
	Chloroform	2	20	risk
	ADVIA cn-free HGB / ADVIA defoamer	3 /3	400/400	
Local respiratory effect	Methylene Blue	2	60	
	Hydrochloric acid	2	40	8 % High
	Brilliant Cresyl Blue	2	40	risk
	White diff	2	40	

The chemical reagents, namely ADVIA perox 1, ADVIA perox 2, ADVIA perox 3, ADVIA shealth, Fuchine basique, Gene expert, Noir soudan B, and Phénol aqueux, are all classified as carcinogens, with a hazard level of 3, representing a high health risk (Table 5).

Table 5. Chemicals with carcinogenic, mutagenic, or reprotoxic (CMR) risks

Chemical Products	Type of risk	Hazard level	Risk index
ADVIA perox 1	Carcinogenic	3	4
ADVIA perox 2	Carcinogenic	3	4
ADVIA perox 3	Carcinogenic	3	4
ADVIA shealth	Carcinogenic	3	4
Chloroform	Carcinogenic	3	200
	Reprotoxic	2	20
Formaldehyde	Carcinogenic	3	600
	Mutagenic	3	600
Basic fuchsin	Carcinogenic	3	6
Gene expert	Mutagenic	3	2
Sudan Black B	Mutagenic	3	4
Aqueous Phenol	Mutagenic	3	2

The chloroform is doubly classified as a carcinogen (hazard level 3) and toxic for reproduction (hazard level 2). Prolonged or repeated exposure to chloroform increases the risk of developing certain types of cancer, including liver and kidney cancer. Additionally, exposure during pregnancy enhances the risk of complications and

adverse effects on fetal development. It is crucial to take appropriate precautions to minimize exposure to chloroform and thereby reduce these health risks (Dumas & Persoons, 2004; INRS, 2023a).

Formaldehyde is also doubly classified as a carcinogen and mutagen, with a hazard level of 3 for both types of risks. Prolonged exposure to formaldehyde can increase the risk of developing certain types of cancer, including nasopharyngeal and sinus cancer. In addition to the carcinogenic risk, evidence of reproductive toxicity is established in animal studies, including fetal developmental anomalies and a decrease in fertility. It is crucial to take adequate measures to reduce exposure to formaldehyde and thereby minimize these health risks.

Conclusion

This in-depth study on the risk assessment of chemical reagents in hemobiology laboratories in Annaba has revealed significant risks associated with certain commonly used chemical reagents, including formaldehyde, staining reagents, and detergents. These substances have been identified as potentially causing respiratory and skin problems among laboratory personnel.

Recommendations

Our results underscore the vital importance of implementing appropriate safety measures during the handling and use of these chemical reagents in a clinical environment. It is essential to adhere to safety guidelines, wear appropriate personal protective equipment, and minimize exposure to these substances as much as possible to reduce health risks for medical personnel and patients.

Scientific Ethics Declaration

The authors declare that the scientific ethical and legal responsibility of this article published in EPHELS journal belongs to the authors.

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References

Berrubé, A., Mosqueron, L., Cavereau, D., Gangneux, J.-P., & Thomas, O. (2013). Méthodologie d'évaluation semi-quantitative du risque chimique en établissement de soins. *Environnement, Risques & Santé, 12*(6), 508-520.

CNRACL. (2007). *Evaluation des Risques Chimiques en établissements de santé*. Réseau inter-CHU d'échange et de mutualisation des informations en médecine du travail des personnels des établissements de santé.

Dumas, L., & Persoons, R. (2004). *Évaluation des risques toxiques professionnels dans les laboratoires du CHU de Grenoble*.(Doctoral dissertation). Médecine humaine et pathologie, Universite Joseph Fourier.

Baurès E. (2016). La qualité de l'air intérieur dans les hôpitaux. *Les cahiers de la Recherche : Santé, Environnement, Travail, (8)*,50-51.

INRS. (2023a). *Agents chimiques CMR. Santé et sécurité autravail INRS*. Retrieved from https://www.inrs.fr/

INRS. (2023b). *Risques chimiques : Évaluation et prévention des risques chimiques*. *Santé et sécurité autravail.* INRS. Retrieved from https://www.inrs.fr/

INRS. (2023c). Risques chimiques. Protection individuelle contre les risques chimiques. Santé et sécurité autravail. INRS. Retrieved from https://www.inrs.fr/

Persoons, R., Dumas, L., Stoklov, M., & Maître, A. (2008). Développement d'une nouvelle méthode d'évaluation des risques chimiques : Application dans les laboratoires hospitaliers. *Archives des maladies professionnelles et de l'environnement .EM-Consulte*, (4), 326-334.

Vincent, R., Bonthoux, F., Mallet, G., Iparraguirre, J. F., & Rio, S. (2005). Méthodologie d'évaluation simplifiée du risque chimique : Un outil d'aide à la décision. *Hygiène et sécurité du travail*, (*200*), 39-62.

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Selective Screening for Inborn Errors of Metabolism Using Tandem Mass Spectrometry in Newborns of the West Kazakhstan: Pilot Study

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Abstract: Tandem mass spectrometry can detect and quantify many metabolites in a single blood spot to diagnose amino acid disorders, organic acids, fatty acid oxidation, and urea cycle disorders. The use of tandem mass spectrometry (MS/MS) is expanding for the implementation of newborn screening programs for inborn errors of metabolism and for selective screening of children of different ages. In Kazakhstan, the use of MS/MS in metabolic screening programs is not yet developed due to the high cost of equipment and consumables and the lack of special screening centers and specialists. Data on the prevalence of most inborn errors of metabolism in Kazakhstan are not presented in the literature. Aim: to perform selective screening for hereditary metabolic diseases among newborns in western Kazakhstan using the liquid chromatography-tandem mass spectrometry (LC-MS/MS) method. Methods: Selective screening was performed among 250 newborns with suspected hereditary metabolic disorders using tandem mass spectrometry. Results: The results of selective newborn screening were interpreted by comparison with reference values established for this group. Diagnosis was based on clinical signs, blood levels of amino acids, acylcarnitines, succinylacetone, urine organic acids, and gene mutation tests. An assessment of 37 inborn errors of metabolism frequencies in high-risk newborns was performed. Conclusion: The research will further develop the national as selective as expanded newborn screening programs.

Keywords: Inborn errors of metabolism, Tandem mass spectrometry, Screening, Newborns

Introduction

Selective screening is an essential tool for diagnosing various types of inborn errors of metabolism (IEM). IEM are a group of phenotypically and genotypically heterogeneous metabolic disorders caused by mutations in genes encoding enzymes of metabolic pathways or receptors. Deficiency or change in the activity of necessary enzymes or other proteins in intermediate metabolic pathways leads to the accumulation or deficiency of the corresponding metabolites in cells or body fluids, manifesting in a wide range of diseases with clinical heterogeneity, thus complicating their diagnosis (Mak et al., 2013). Many IEM do not have specific clinical signs and are difficult to diagnose using only clinical manifestations or routine laboratory tests (Champion et al., 2010). Most often, IEM occur in early infancy and childhood, and the prevalence within different racial and ethnic groups is not the same. Hence, there are population differences in the incidence of IEM (Champion et al., 2010; Lampret et al., 2015; Shibata et al., 2018; Sarker et al., 2019). Tandem mass spectrometry can detect and quantify many metabolites in a single blood spot to diagnose amino acid disorders, organic acids, fatty acid oxidation, and urea cycle disorders (Chace et al., 2009; Lampret et al., 2015; Kaysheva et al., 2022; Gelb et al., 2022). The use of tandem mass spectrometry is expanding for the implementation of newborn screening programs (NBS) for inborn errors of metabolism and for selective screening of children of different ages (Landau et al., 2017; Shibata et al., 2018). The results from these expanded NBS programs provided information

on the prevalence of these diseases in the USA (Landau et al., 2017), some countries in Europe (Scolamiero et al., 2015; Messinaet al., 2018; Lampretet al., 2020; Loeber et al., 2021), and Asia (Yunus et al., 2016; Yang et al., 2020; Deng et al., 2021).

In Kazakhstan, the use of MS/MS in metabolic screening programs is not yet developed due to the high cost of equipment and consumables and the lack of special screening centers and specialists. Data on the prevalence of most inborn errors of metabolism in Kazakhstan are not presented in the literature.

Study Objectives

To perform selective screening for hereditary metabolic diseases among newborns with suspected IEM in western Kazakhstan using the LC-MS/MS method.

Tasks

1. to assess the burden of metabolic disorders detected by LC-MS/MS in western Kazakhstan by examination of newborns at clinical risk in pediatric clinics throughout the region;
2. to analyze prevalence, and age of onset for each identified IEM, further comparing the obtained findings with those from previously published reports in other populations.

Methods

Study Design and Ethics

In this observational study, a cross-sectional design is used due to its screening nature. The data for the present research will be derived from a selective LC-MS/MS IEM screening of 250 clinical-risk newborns.

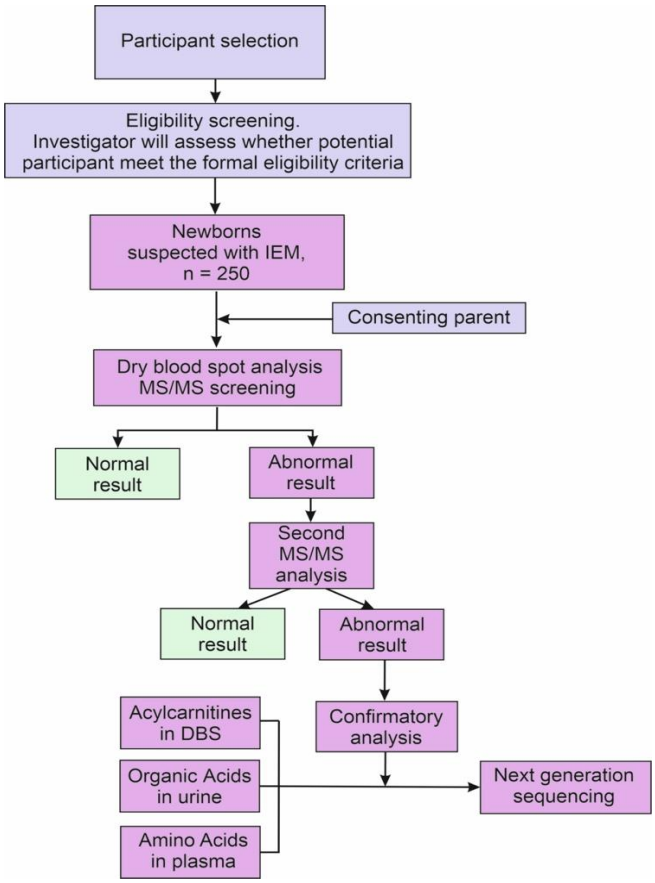

Figure 1. Flowchart of the study. (DBS means Dried Blood Spot analysis)

This research poses no risk to participating individuals. All study procedures are conducted according to the principles of the Declaration of Helsinki (2013), and patient rights are observed. Participation in the study is voluntary. Informed consent is obtained from all parents and/or legal guardians of children involved in the study. The study was approved by the Bioethics Committee of the West Kazakhstan Marat Ospanov Medical University (Ref. No. 7, 09/09/2020.).

Criteria for Inclusion in the Study

The inclusion criteria for IEM selective screening are presented in Table 1.

Table 1. Criteria for inclusion in the selective screening for IEM.

Main criteria (symptoms)	Sudden deterioration in the clinical condition of the child after a period of normal development (days, weeks, months): acute metabolic encephalopathy lethargy (coma) seizures resistant to antiepileptic therapy Hepatomegaly (hepatosplenomegaly) Metabolic acidosis with increased anion gap Multiple fractures Child mortality in the family from diseases with similar symptoms
Additional criteria (symptoms)	Treatment-resistant seizures Abnormal muscle tone: dystonia, hyperkinesis, hypotension Speech retardation Mental retardation of unknown cause Cardiomyopathy Tachypnea Frequent spitting up (vomiting) Osteoarticular anomalies (joint stiffness, chest deformity, rickets-like changes) Hernias (umbilical, inguinal-scrotal) Persistent or recurrent hypoglycemia Metabolic alkalosis An increase in ketone bodies in the blood and (or) urine Hyperammonemia An increase in the level of liver enzymes (ALAT, AST) more than 1.5 times Increase in the level of creatine phosphokinase (CPK) by more than 2 times Decrease in the level of alkaline phosphatase (AP) below the age norm Imaging or electrophysiological examinations indicating a metabolic disorder Leukopenia Thrombocytopenia Abnormal odor of urine, body, earwax, any unusual odor Hair growth disorders, alopecia Ophthalmic anomalies Unusual appearance, dysmorphic features History of death of a previous sibling of unknown cause Parents' consanguinity Positive family history with metabolic disorders

Exclusion criteria:

Patients having the following conditions will be excluded:

1. perinatal brain injury,
2. brain injury,
3. infections of the central nervous system,
4. toxicological diseases,
5. tumors,
6. chromosomal abnormalities,
7. symptoms specified in the Inclusion criteria, but with a confirmed diagnosis of any disease other than amino acid disorders (AAD), fatty acid oxygenation disorders (FAOD), or organic acidemias (OA).

Mass Spectrometry Analysis

Specimen Collection and Storage

Neonatal whole blood samples were collected from infants no earlier than 3 hours after feeding by heel prick using a heel stick. Five drops of whole blood (each ~75 µl) were applied to Guthrie cards, Ahlstrom 226 filter paper, and PerkinElmer 226 Five-Spot Card (PerkinElmer Health Sciences, Greenville, USA) to form dried blood spots (DBSs) for LC-MS/MS analysis. Samples were dried for 4 hours at room temperature and then stored at 4°C in labeled individual zip-lock plastic envelopes with desiccants until analyzed by LC-MS/MS. Samples were sent to the laboratory within five days. In the case of long-term storage of samples, it was carried out at a temperature of –20°C.

Specimen Preparation and LC-MS/MS Analysis

The Neobase2 TM Non-derivatized MSMS kit (PerkinElmer, Wallac Oy, Turku, Finland) was used to quantify 15 amino acids, free carnitine, 35 acylcarnitines, and succinylacetone in DBS according to the manufacturer's instructions. Metabolites to be measured: Amino acids: Alanine (Ala), Arginine (Arg), Citrulline (Cit), Glutamine (Gln), Glutamic acid (Glu), Glycine (Gly), Leucine (Leu), Isoleucine (Ile), Hydroxyproline (Pro-OH), Methionine (Met), Ornithine (Orn), Phenylalanine (Phe), Proline (Pro), Tyrosine (Tyr), Valine (Val).

Acylcarnitines: Free carnitine (C0), Acetylcarnitine (C2), Propionylcarnitine (C3), Malonylcarnitine+3-Hydroxybutyrylcarnitine (C3DC/C4OH), Butyrylcarnitine (C4), 2H9-C5-Methylmalonylcarnitine+3-Hydroxyisovalerylcarnitine (C4DC/C5OH), Isovalerylcarnitine (C5), Tiglylcarnitine (C5:1), Glutarylcarnitine (C5DC), Hexanoylcarnitine (C6), Octanoylcarnitine (C8), Octenoylcarnitine (C8:1), Decanoylcarnitine (C10), Decenoylcarnitine (C10:1), Decadienoylcarnitine (C10:2), Dodecanoylcarnitine (C12), Hydroxydodecenoylcarnitine (C12:1), Myristoylcarnitine (C14), Tetradecenoylcarnitine (C14:1), Tetradecadienyl-carnitine (C14:2), Hydroxytetradecanoylcarnitine (C14OH), Palmitoylcarnitine (C16), Hexadecenoylcarnitine (C16:1), 2H3-C16-3-Hydroxy-Hexadecanoylcarnitine (C16OH), 2H3-C16-3-Hydroxypalmitoleylcarnitine (C16:1OH), 2H3-Stearoylcarnitin (C18), 2H3-C18-Octadecenoylcarnitine (C18:1), 2H3-C18-Linoleylcarnitine (C18:2), 2H3-C18-3-Hydroxystearoylcarnitine (C18OH), 2H3-C18-3-Hydroxyoleoylcarnitine (C18:1OH), 2H3-C18-3-Hydroxylinoleoylcarnitine (C18:2OH).
Succinylacetone (SUAC) (13C5-MPP IS).

DBS were analyzed using a Shimadzu LCMS-8050 Triple Quadrupole Mass Spectrometer (Shimadzu Corporation, Kyoto, Japan). Sample preparation was based on extraction followed by derivatization into oil esters. Level I and Level II (low standard and high standard) dried blood drops were included with each assay lot of the Neobase2 TM Non-derivatized MSMS kit to monitor system accuracy and precision. To analyze amino acids and acylcarnitines, stored DBS card samples are brought to room temperature (+18 to +25°C) before extraction. A 3.2 mm disc (equivalent to ~3.1 µl of whole blood) is punched out of one dried blood spot with a diameter of 3.2 mm using a Wallac DBS Puncher (PerkinElmer, Wallac Oy, Mustionkatu 6, FI-20750 Turku, Finland) into the well of the 96-well polystyrene U-bottom microplate supplied with the Neobase2 TM Non-derivatized MSMS kit. After adding 125 µL of working extraction solution to each well of the microplate, the plate is covered with an adhesive aluminum film and incubated for 30 minutes at room temperature on a microplate shaker with a shaking speed of 650 rpm. After incubation, 100 µL of the supernatant is transferred to a new 96-well U-bottom microplate, covered with aluminum foil to reduce evaporation, and incubated for 1 hour. The plate is then placed into the Shimadzu LCMS-8050 Triple Quadrupole Mass Spectrometer autosampler, and 5 µL of supernatant is injected into the LCMS for analysis.

Statistical Analysis

Shapiro-Wilk and Kolmogorov-Smirnov tests were used to check the normality of the distribution. The data obtained in the study demonstrated that the distribution of amino acids and acylcarnitines in DBS differs from normal. Me (median) and quartiles (IQR interquartile range) were used for descriptive statistics of the samples. Nonparametric tests (Mann-Whitney U test, Kruskal-Wallis H test) were used to test differences in amino acids and acylcarnitines concentrations depending on various factors (gender, place of residence). Two-sided levels <0.05 are assumed to be statistically significant. Statistical analysis will be carried out using the statistical packages IBM SPSS v. 23.0 (IBM, Armonk, NY, USA), Statistica (StatSoft, Inc., Tulsa, OK, USA, v. 10), and R 3.3.2 (R Foundation for Statistical Computing, Vienna, Austria).

Results and Discussion

The study is currently in the recruitment stage. In total, samples from 130 study participants were collected. Demographic and anthropometric data of newborns with suspected hereditary metabolic diseases are presented in Table 2.

Table 2. Demographic and anthropometric data of newborns with suspected hereditary metabolic diseases

	Newborns with suspected IEM (n=130)
Weight in grams, Median (IQR)	3007 (2522;3489)
Gender	
Male, n, %	66 (50.8 %)
Female, n, %	64 (49.2 %)
Geographic distribution	
Urban population, n, %	89 (68.5 %)
Rural population, n, %	41 (31.5 %)

Table 3 describes the primary clinical manifestations and main diagnostic markers of IEM and their frequency in high-risk newborns. Currently, these clinical manifestations have not been confirmed by LC-MS/MS in the majority of children examined. Only a small proportion of patients (5) showed deviations from the reference values of amino acids and acylcarnitines established earlier in our study.

Table 3. Common features encountered in newborns with suspected IEM (n = 130).

Variables	Number of patients (percent)
Developmental delay	78 (60.0 %)
Neurological abnormalities	82 (63.1 %)
Disturbed consciousness level	8 (6.15 %)
Vomiting/dehydration	13 (10.0 %)
Hyperammonemia	11 (8.46 %)
Metabolic acidosis	24 (18.5 %)
MRI brain abnormalities	7 (5.38 %)
Infections	61 (46.9 %)
Hypoglycemia	15 (11.5 %)
Organomegaly	9 (6.92 %)
Micro/macrocephaly	38 (29.2 %)
Ophthalmic abnormalities	7 (5.38 %)
Cardiopathy	18 (13.8 %)
Seizures	48 (36.9 %)
Tachypnea	24 (18.5 %)
Thrombocytopenia	6 (4.6 %)
Abnormal smell	1 (0.07 %)
Hair growth disorders	6 (4.61 %)
Osteoarticular anomalies	17 (13.08 %)
Abnormal muscle tone	82 (63.1 %)

Conclusion

The data of selective screening conducted among newborns at high risk of IEM, given the inclusion in the study of the largest pediatric hospitals in western Kazakhstan, can serve as a basis for calculating the relative frequencies of various IEMs in the child population of the region. The research will further develop the national as selective as expanded newborn screening programs.

Scientific Ethics Declaration

The authors declare that the scientific ethical and legal responsibility of this article published in EPHELS Journal belongs to the authors.

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* The authors declare no conflict of interest.

References

Chace, D. H. (2009). Mass spectrometry in newborn and metabolic screening: historical perspective and future directions. *Journal of Mass Spectrometry: JMS*, *44*(2), 163–170.

Champion, M. P. (2010). An approach to the diagnosis of inherited metabolic disease. *Archives of Disease in Childhood Education and Practice Edition*, *95*(2), 40–46.

Deng, K., Zhu, J., Yu, E., Xiang, L., Yuan, X., Yao, Y., Li, X., & Liu, H. (2021). Incidence of inborn errors of metabolism detected by tandem mass spectrometry in China: A census of over seven million newborns between 2016 and 2017. *Journal of Medical Screening*, *28*(3), 223–229.

Gelb, M. H., Basheeruddin, K., Burlina, A., Chen, H. J., Chien, Y. H., Dizikes, G., Dorley, C., Giugliani, R., Hietala, A., Hong, X., Kao, S. M., Khaledi, H., Klug, T., Kubaski, F., Liao, H. C., Martin, M., Manning, A., Orsini, J., Peng, Y., Ranieri, E., …& Matern, D. (2022). Liquid chromatography-tandem mass spectrometry in newborn screening laboratories. *International Journal of Neonatal Screening*, *8*(4), 62.

Kaysheva, A. L., Kopylov, A. T., Stepanov, A. A., Malsagova, K. A., Izotov, A. A., Shurubor, Y. I., & Krasnikov, B. F. (2022). Chromatomass-spectrometric method for the quantitative determination of amino- and carboxylic acids in biological samples. *Metabolites*, *13*(1), 16.

Lampret, B. R., Murko, S., Tanšek, M. Ž., Podkrajšek, K. T., Debeljak, M., Šmon, A., & Battelino, T. (2015). Selective screening for metabolic disorders in the slovenian pediatric population. *Journal of Medical Biochemistry*, *34*(1), 58–63.

Lampret, B. R., Remec, Ž. I., Torkar, A. D., Tanšek, M. Ž., Šmon, A., Koračin, V., Čuk, V., Perko, D., Ulaga, B., Jelovšek, A. M., Debeljak, M., Kovač, J., Battelino, T., & Grošelj, U. (2020). Expanded newborn screening program in Slovenia using tandem mass spectrometry and confirmatory next generation sequencing genetic testing. *Zdravstveno Varstvo*, *59*(4), 256–263.

Landau, Y. E., Waisbren, S. E., Chan, L. M., & Levy, H. L. (2017). Long-term outcome of expanded newborn screening at Boston children's hospital: benefits and challenges in defining true disease. *Journal of Inherited Metabolic Disease*, *40*(2), 209–218.

Loeber, J. G., Platis, D., Zetterstrom, R. H., Almashanu, S., Boemer, F., Bonham, J. R., Borde, P., Brincat, I., Cheillan, D., Dekkers, E., Dimitrov, D., Fingerhut, R., Franzson, L., Groselj, U., Hougaard, D., Knapkova, M., Kocova, M., Kotori, V., Kozich, V., Kremezna, A., … Schielen, P. C. J. I. (2021). Neonatal screening in Europe revisited: An ISNS perspective on the current state and developments since 2010. *International Journal of Neonatal Screening*, *7*(1), 15.

Mak, C. M., Lee, H. C., Chan, A. Y., & Lam, C. W. (2013). Inborn errors of metabolism and expanded newborn screening: review and update. *Critical Reviews in Clinical Laboratory Sciences*, *50*(6), 142–162.

Messina, M., Meli, C., Raudino, F., Pittalá, A., Arena, A., Barone, R., Giuffrida, F., Iacobacci, R., Muccilli, V., Sorge, G., & Fiumara, A. (2018). Expanded newborn screening using tandem mass spectrometry: seven years of experience in eastern sicily. *International Journal of Neonatal Screening*, *4*(2), 12.

Sarker, S. K., Islam, M. T., Biswas, A., Bhuyan, G. S., Sultana, R., Sultana, N., Rakhshanda, S., Begum, M. N., Rahat, A., Yeasmin, S., Khanam, M., Saha, A. K., Noor, F. A., Sajib, A. A., Islam, A. B. M. M. K., Qadri, S. K., Shahidullah, M., Mannan, M. A., Muraduzzaman, A. K. M., Shirin, T., … & Mannoor, K. (2019). Age-specific cut-off values of amino acids and acylcarnitines for diagnosis of inborn errors of metabolism using liquid chromatography tandem mass spectrometry. *BioMed Research International*, *2019*, 3460902.

Scolamiero, E., Cozzolino, C., Albano, L., Ansalone, A., Caterino, M., Corbo, G., di Girolamo, M. G., Di Stefano, C., Durante, A., Franzese, G., Franzese, I., Gallo, G., Giliberti, P., Ingenito, L., Ippolito, G., Malamisura, B., Mazzeo, P., Norma, A., Ombrone, D., Parenti, G., … Ruoppolo, M. (2015). Targeted metabolomics in the expanded newborn screening for inborn errors of metabolism. *Molecular BioSystems*, *11*(6), 1525–1535.

Shibata, N., Hasegawa, Y., Yamada, K., Kobayashi, H., Purevsuren, J., Yang, Y., Dung, V. C., Khanh, N. N., Verma, I. C., Bijarnia-Mahay, S., Lee, D. H., Niu, D. M., Hoffmann, G. F., Shigematsu, Y., Fukao, T., Fukuda, S., Taketani, T., & Yamaguchi, S. (2018). Diversity in the incidence and spectrum of organic acidemias, fatty acid oxidation disorders, and amino acid disorders in Asian countries: Selective screening vs. expanded newborn screening. *Molecular Genetics and Metabolism Reports*, *16*, 5–10.

Yang, C., Zhou, C., Xu, P., Jin, X., Liu, W., Wang, W., Huang, C., Jiang, M., & Chen, X. (2020). Newborn screening and diagnosis of inborn errors of metabolism: A 5-year study in an eastern Chinese population. *Clinica Chimica Acta; International Journal of Clinical Chemistry*, *502*, 133–138.

Yunus, Z. M., Rahman, S. A., Choy, Y. S., Keng, W. T., & Ngu, L. H. (2016). Pilot study of newborn screening of inborn error of metabolism using tandem mass spectrometry in Malaysia: outcome and challenges. *Journal of Pediatric Endocrinology & Metabolism: JPEM*, *29*(9), 1031–1039.

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Study of the Antibiotic Resistance Profile of *Klebsiella Pneumonia* in Patients Infected with Covid- 19

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Abstract: Antimicrobial resistance (AMR) is a global public health challenge. AMR is the ability of a microorganism to resist antimicrobial attack, which in turn prevents successful treatment of an infection. Massive antibiotic therapy has led to an increase in the antibiotic resistance of pathogens of infectious diseases. The problem of antibiotic resistance is especially acute in the treatment of nosocomial infections. Nosocomial infections also called “hospital-acquired infections”, are infections acquired during hospital care which are not present or incubating at admission. Infections occurring more than 48 hours after admission are usually considered nosocomial. The main reason for the prolonged hospitalization of patients infected with COVID-19 is to internal infections of the hospital and complications caused by their influence. it was important to determine the bacterial spectrum of etiological agents of nosocomial infections in patients infected with COVID-19 and the profile of antibiotic resistance. The research materials were collected at the different hospital resuscitations from patients undergoing treatment for a long time and who were resistant to antibiotics. As the resalts showed the highest resistance to amp/sulbactam, Aztreonam, ampicillin, meropenem, moxifloxacin, cefepime, ceftazidime, cefotaxime, ciprofloxacin Levofloxacin, amox/clavulanate, norfloxacin, pip/tazobactam, ceftriaxone was detected in 100 %, resistance to cefoxitin, trim/sulfa and amikacin in 90%, resistance to tobramycin in 80%, and the relatively low resistance was detected to gentamycin in 20 %.

Keywords: Klebsiella pneumonia, Antibiotic resistance, Covid- 19, Nosocomial infection

Introduction

The antibiotic resistance profile of *Klebsiella pneumoniae* in COVID-19 patients is an important topic of investigation since secondary bacterial infections frequently aggravate the clinical course of COVID-19. *Klebsiella pneumoniae* is a bacterial pathogen that has been linked to healthcare-associated illnesses and has been linked to pneumonia and other severe diseases. Understanding the antibiotic resistance profile of COVID-19 is critical for successful patient care and infection control. Coronavirus disease of 2019 (COVID-19), the disease caused by infection with the SARS-CoV-2 virus, has caused the worst pandemic since the 1918 influenza. Through the end of October 2022, 627 million confirmed cases and 6.5 million deaths from COVID-

19 have been reported globally. Patients infected by SARS-CoV-2 can be coinfected with Klebsiella pneumoniae while in the intensive care unit (ICU). During the pandemic, the capacity of many ICUs was exceeded due to the large increase in hospital admissions, which could have increased the frequency of co-infection with nosocomial microorganisms such as carbapenemase-producing Enterobacterales (CPE). (Cañada-García et al., 2023). Bacterial and fungal nosocomial infection is a common complication of ICU admission in patients with COVID-19. It usually presents as a severe form of infection, and it is associated with a high mortality and longer course of ICU stay. (Bardi et al., 2021).

The COVID-19 era brought about new medical challenges, which, together with nosocomial bacterial infections, resulted in an enormous burden for the healthcare system. One of the most alarming nosocomial threats was carbapenem-resistant Klebsiella pneumoniae (CRKP). Monitoring CRKP incidence and antimicrobial resistance globally and locally is vitally important. (Ficik et al.,2023).

In recent years, the rapid spread of resistance caused by the production of extended-spectrum beta-lactamase (ESBL) among bacteria has increased the importance of *Klebsiella pneumoniae* bacteria. Regular monitoring of antibiotic resistance rates of bacteria is very important for the treatment of infections and new treatment methods that can be developed. In the study, it was aimed to determine the antibiotic resistance profiles and ESBL positivity rates of *Klebsiella pneumoniae* isolated from patients. (Doganay et al., 2023)

Materials and Methods

Susceptibility profile and identification of the infection *klebsiella pneumonia (*n=12) isolates collected in different hospital services were performed by disc diffusion methods according to the EUCAST guidelines, and API 20E, respectively. The research materials were collected at the different hospital resuscitations from patients undergoing treatment for a long time and who were resistant to antibiotics. The biological fluid, urine, blood and sputum were used as the study materials. Within the grant have been done (Targeted scientific research projects of BSU) - Collecting nosocomial infections in suspected patients in the resuscitation of hospitals in Adjara; Doing bacteriological research of a sample to determine the possible causative agents in the material, the isolation using the method of pure culture, the plating of a sample on the various media; Selecting, staining and microscopy of suspicious colonies on the basis of the first examination of the Petri dishes; Obtaining pure cultures by subculturing; The final identification of the microorganisms by using the identification systems Api- 20E.

Antimicrobial agents (amp/sulbactam, cefoxitin, Aztreonam, ampicillin, meropenem, moxifloxacin, cefepime, trimethoprim–sulfamethoxazole, tobramycin, ceftazidime, cefotaxime, ciprofloxacin, Levofloxacin, amox/clavulanate, norfloxacin, piperacillin–tazobactam, gentamicin, ceftriaxone, imipenem, amikacin) for *Klebsiella pneumoniae* were used for AST. Because of differences in the concentrations of some antimicrobial agents between the EUCAST , BD BBL Sensi-Disc (Becton, Dickinson and Company) and Thermo Scientific Oxoid disc (Oxoid, Basingstoke, UK) were selectively used according to the EUCAST (European Committee on Antimicrobial) Susceptibility Testing (EUCAST) guidelines. For *Klebsiella pneumoniae* (gram-negative bacilli), disk diffusion tests were performed using Oxoid disks containing piperacillin–tazobactam, cefotaxime, and ceftazidime and BD disks containing imipenem, meropenem, ciprofloxacin, amikacin, gentamicin, tobramycin, and trimethoprim–sulfamethoxazole.

The antibiotic resistance of klebsiella *pneumonia* to 19 various antibiotics was determined. The test of antibiotic resistance clearly showed a high resistance. Disk diffusion method was employed to evaluate antimicrobial susceptibility against amp/sulbactam, cefoxitin, Aztreonam, ampicilin, meropenem, moxifloxacin, cefepime, trimethoprim–sulfamethoxazole, tobramycin, ceftazidime, cefatoxim, ciprofloxacin, Levofloxacin, amox/clavulanate, norfloxacin, piperacillin–tazobactam, gentamicin, ceftriaxon, imipenem, amikacin.

Results and Discussion

As the results of microbiological research (by using the disc diffusion method of antibiotic sensitivity, the method of carbapenem resistance, ESBL testing method), it has been found that antibiotic resistance condition is enough severe. Antibiotics of different generations, used in this study, have shown a high-quality of resistance, which should be considered. Antibiotic sensitivity was studied by using the disc diffusion method where the variety of antibiotics has been used. The indicators of sensitivity for each microbe were calculated according to the regulation European Committee on Antimicrobial Susceptibility Testing (EUCAST).

meropenem, moxifloxacin, cefepime, ceftazidime, cefotaxime, ciprofloxacin, Levofloxacin, amox/clavulanate, norfloxacin, pip/tazobactam, ceftriaxone, was detected in 100 %, resistance to cefoxitin, trim/sulfa and amikacin in 90%, resistance to tobramycin in 80%, and the relatively low resistance was detected to gentamycin in 20 % (Figure 1 & Figure 2).

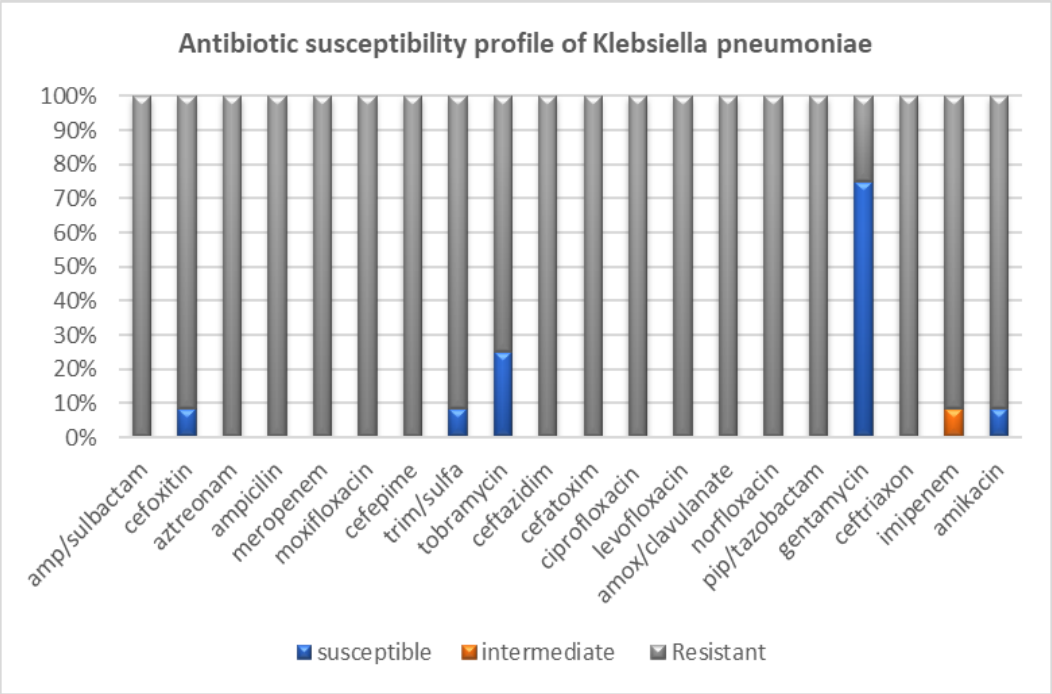

Figure 1. Antibiotic susceptibility profile of *Klebsiella pneumonia*

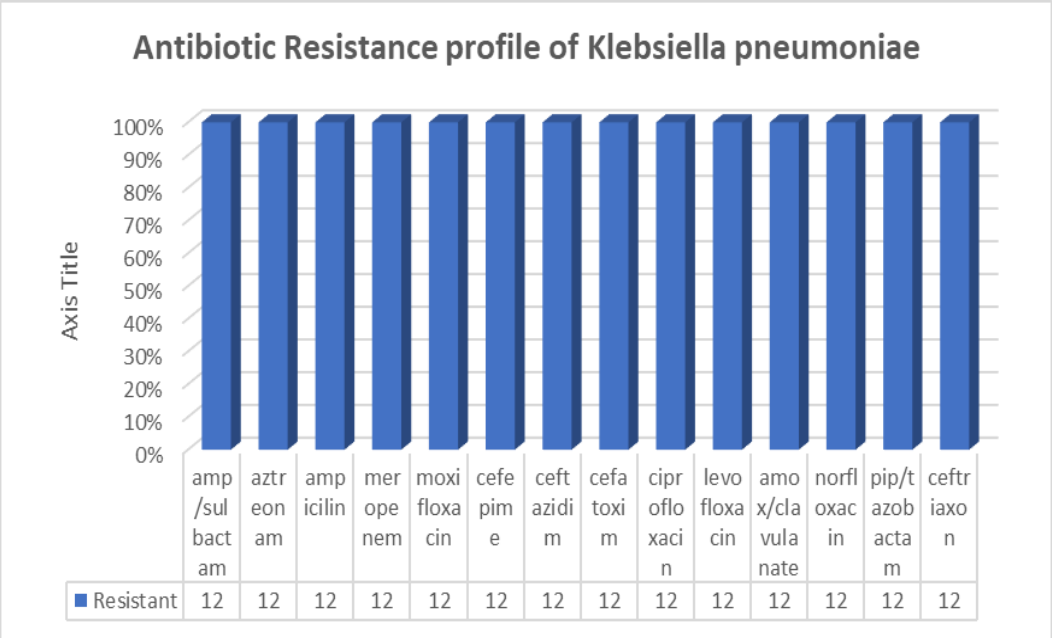

Figure 2. Antibiotic resistance profile of *Klebsiella pneumoniae*

The reasons to develop the antibiotic resistance should be - Making mistakes associated with antibiotic therapy carried out for respiratory infections; Taking an antibiotic without diagnosing the disease of the bacterial origin; The irreducibility of the local situation of resistance in prescribing a medicine; Controlling the antibiotics taken by patients all their life; Starting the antibiotic therapy in the wrong dose; Taking the insufficient sample for the microbiological research; Making an improper evaluation of the severity of infection; The unjustified change of antibiotic; The long or short course antibiotic therapy; The irreducibility of the side effects of antibiotics; Using

the antibiotics, which can not penetrate deep into the tissue; Choosing the wrong antibiotic; Giving the wrong dose of antibiotics.

Conclusion

The COVID-19 pandemic has underscored the importance of addressing nosocomial infections within healthcare settings. Combating the silent threat of healthcare-associated infections requires a coordinated effort, with healthcare facilities, staff, and policymakers working together to implement and enforce infection control measures. As the world continues to navigate the challenges of the pandemic, understanding and mitigating the risk of nosocomial infections is crucial to protect both patients and healthcare workers.

The result of the current study showed the growing number of nosocomial infections associated with *klebsiella pneumonia Resistant* strains increasingly cause public health problems; therefore, their early detection is essential for healthcare centers. It is important to determine not spontaneous, but the correct course of antibiotic therapy, which will be the key to the patient's recovery. The bacterial spectrum of nosocomial infections identified during the study and specific antibiotics that are sensitive to the microbes that cause these infections will allow healthcare workers to properly deal with the infection and, most importantly, save the patient's life. Therefore, the study of this issue on a regional scale is very important.

Antimicrobial resistance (AMR) is a global public health challenge. AMR is the ability of a microorganism to resist antimicrobial attack, which in turn prevents successful treatment of an infection. Massive antibiotic therapy has led to an increase in the antibiotic resistance of pathogens of infectious diseases. The main reason for the prolonged hospitalization of patients infected with COVID-19 is to internal infections of the hospital and complications caused by their influence. it was important to determine the bacterial spectrum of etiological agents of nosocomial infections in patients infected with COVID-19 and the profile of antibiotic resistance. The research materials were collected at the different hospital resuscitations from patients undergoing treatment for a long time and who were resistant to antibiotics.

Scientific Ethics Declaration

The authors declare that the scientific ethical and legal responsibility of this article published in EPHELS journal belongs to the authors.

Acknowledgements or Notes

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References

Bardi, T., Pintado, V., Gomez-Rojo, M., Escudero-Sanchez, R., Azzam Lopez, A., Diez-Remesal, Y., ... & Pestaña, D. (2021). Nosocomial infections associated to COVID-19 in the intensive care unit: clinical characteristics and outcome. *European Journal of Clinical Microbiology & Infectious Diseases*, *40*, 495-502.

Cañada-García, J. E., Ramírez de Arellano, E., Jiménez-Orellana, M., Viedma, E., Sánchez, A., Alhambra, A., & Oteo-Iglesias, J. (2023). Carbapenemase-Producing Klebsiella pneumoniae in COVID-19 intensive care patients: Identification of IncL-VIM-1 plasmid in previously non-predominant sequence types. *Antibiotics*, *12*(1), 107.

Doganay, D., Aydin, M., & Avsar I. (2023). Investigation of the antıbıotıc resıstance profile of klebsıella pneumonıae isolates ısolated from dıfferent clınıcal specimens: *One-Year Data Durıng The Covıd-19 Pandemıc Process. 47*(1),185-195.

Falcone, M., Tiseo, G., Arcari, G., Leonildi, A., Giordano, C., Tempini, S., ... & Menichetti, F. (2022). Spread of hypervirulent multidrug-resistant ST147 Klebsiella pneumoniae in patients with severe COVID-19: an observational study from Italy, 2020–21. *Journal of Antimicrobial Chemotherapy*, *77*(4), 1140-1145.

Ficik, J., Andrezál, M., Drahovská, H., Bohmer, M., Szemes, T., Liptáková, A., & Slobodníková, L. (2023). Carbapenem-resistant Klebsiella pneumoniae in COVID-19 era—challenges and solutions. *Antibiotics*, *12*(8), 1285.

Gabriele Arcari 1, G. R. (2021). Klebsiella pneumoniae infections in COVID-19 patients: a 2-month retrospective analysis in an Italian hospital. *Int J Antimicrob Agents, (1)*,106245.

Ipek, S., Sahin, A., Gungor, S., Yurttutan, S., Gullu, U. U., Inal, S., ... & Demiray, S. D. (2022). Nosocomial infections in non-Covid-19 pediatric patients prior to and during the pandemic in a pediatric ıntensive care unit. *Cureus*, *14*(1). e21451.

Lai, C. C., Chen, S. Y., Ko, W. C., & Hsueh, P. R. (2021). Increased antimicrobial resistance during the COVID-19 pandemic. *International Journal of Antimicrobial Agents*, *57*(4), 106324.

Onal, U., Tuzemen, U., Kazak, E., Gencol, N., Souleiman, E., Imer, H., ... & Akalin, H. (2023). Effects of COVID-19 pandemic on healthcare-associated infections, antibiotic resistance and consumption rates in intensive care units. *Le Infezioni in Medicina*, *31*(2), 195.

O'Toole, R. F. (2021). The interface between COVID-19 and bacterial healthcare-associated infections. *Clinical Microbiology and Infection*, *27*(12), 1772-1776.

Pourajam, S., Kalantari, E., Talebzadeh, H., Mellali, H., Sami, R., Soltaninejad, F., ... & Solgi, H. (2022). Secondary bacterial infection and clinical characteristics in patients with COVID-19 admitted to two intensive care units of an academic hospital in Iran during the first wave of the pandemic. *Frontiers in Cellular and Infection Microbiology*, *12*, 141.

Saeed, N. K., Al-Khawaja, S., Alsalman, J., Almusawi, S., Albalooshi, N. A., & Al-Biltagi, M. (2021). Bacterial co-infection in patients with SARS-CoV-2 in the Kingdom of Bahrain. *World Journal of Virology*, *10*(4), 168.

Tang, H., Zhao,Z., Zhang, X., Pan, L., Wu, Q., Wang, M.,…& Li, F.(2021). Analysis of pathogens and risk factors of secondary pulmonary infection in patients with COVID-19. *Microbial Pathogenesis*, *156*, 104903.

Tiri, B., Sensi, E., Marsiliani, V., Cantarini, M., Priante, G., Vernelli, C., ... & Cappanera, S. (2020). Antimicrobial stewardship program, COVID-19, and infection control: spread of carbapenem-resistant Klebsiella pneumoniae colonization in ICU COVID-19 patients. What did not work?. *Journal of Clinical Medicine*, *9*(9), 2744.

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ECG Tools for Cancer Screening

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Abstract: Various cancers can be screened quickly by detection of visible and invisible abnormal findings appearing at ECGs. Data were statistically processed using variation and regression analyses. Evaluation of statistical reliability for the groups studied was made according to the p-value for the meaning of chi-square, and differences were considered significant at $p<0.05$. In the group was included 31 individuals without cancer and 67 persons with different types of cancer. p- wave may be use with an insignificantly degree of probability as a predictor of cancer for women. QRS complex may be used as an additional indicator of cancer for men. Dividing the groups by sex showed the presence of statistically significant difference between the mathematical expectations for the groups. The results showed that obtained logistic regression model possessed good abilities for cancer prediction among men, based on the ECG.

Keywords: ECG, Tools, Cancer, Screening, Prediction

Introduction

New tools for cancer screening covers a broad spectrum of innovations including optical sensors, nanotechnology, affinity agents, imaging contrast agents, nanofluidics and cell-based assays. Detection of cancers by non-invasive methods such as X-Ray, CT scan, and MRI & PET scan are non-invasive and quick but very expensive. The following are examples of non-invasive quick method of diagnosis and treatment of cancers using different approaches:

- Soft red laser beam scanning of different parts of body;
- By speaking voice;
- Using strong electromagnetic field resonance phenomenon between 2 identical molecules or tissues, known as O-Ring Test, for which US patent was given, we can identify any molecules non-invasively. Using this method, we are able to map accurate organ representation areas at different parts of the body surfaces.

Objectives

Objectives of this study are to develop new non-invasive, safe, quick and economical method of detecting cancers by ECGs.

Method

Data were statistically processed using variation and regression analyses. Evaluation of statistical reliability for the groups studied was made according to the p-value for the meaning of chi-square, and differences were considered significant at $p<0.05$.

ANOVA Analysis

ANOVA analysis was made of different factors through dividing all persons investigated into groups. The participants were divided into four groups: group 1
– males who had cancer, group 2 – males without cancer, group 3 – females who had cancer, and group 4 – females without cancer.

Logistic Regression Analysis

Regression analysis is applied to describe the dependence between one dependent variable and one or more independent variables. The odds ratio is used as a measure for the degree of dependence between risk factors and cancer. Logistic regression analysis may help to find the most appropriate and cost- effective, as well as the most acceptable model, which can describe the relationship between the outcome of a disease and a multitude of independent variables (factors).

Participants

In the group was included 31 individuals without cancer and 67 persons with different types of cancer from Northwest Bulgaria (Table 1).

Table 1. Individuals with cancer

№	Diagnosis	men	women
1.	Colon cancer	5	2
2.	Hypopharyngeal cancer	1	-
3.	Pancreatic cancer	2	2
4.	Lung cancer	6	3
5.	Rectal cancer	6	1
6.	Kydney cancer	1	-
7.	Sigma cancer	3	1
8.	Stomach cancer	1	-
9.	Bladder cancer	1	-
10.	Prostate cancer	6	-
№	Diagnosis	men	women
11	Metastatic cancer	1	-
12	Schloffer-Tumor	1	-
13	Uterine cancer	-	4
14	Endometrial cancer	-	3
15	Breast cancer	-	8
16	Ovarian cancer	-	5
17	Follicular lymphoma (form of non- Hodgkin`s lymphoma)	-	1
18	Mantle cell lymphoma (form of non-Hodgkin`s lymphoma)	-	1
19	Metastatic cancer	-	1
20	Sigma polypus	-	1

A total of 67 patients with cancer were selected from 98 participants. The number of women with cancer was 33 and the number of men was 34. For the study, 67 ECGs of oncology patients, which were collected at the Department of Oncology from July 2017 to April 2018, were provided by Pleven University Hospital(Bulgaria). All data and samples derived from the University Hospital of Pleven were obtained with informed consent under Institutional Review Board. 31 ECGs of patients undergoing surgery without any tumors were collected at the Department of Surgery of Pleven University Hospital.

Heart rate was 77.53/min for oncology patients and 81.24/min for other people in the study.

Systolic (SBP) blood pressure, diastolic (DBP) blood pressure and BMI were measured.
The 67 serum samples of patients with different tumors were evaluated for CBC. We also collected 31 serum samples from 31 patients without cancer as controls in April 2018. The number of women was 17 and the number of men was 14.

Table 2. Basic clinical characteristic of groups

Characteristic	Individuals with cancer		Individuals without cancer	
	Mean value	SD	Mean value	SD
Age	64	±12	56	±17
DBP [mm Hg]	74,93	±5.87	76.13	±9.89
SBP [mm Hg]	122,91	±7.13	122.74	±10.94
Weight [kg]	72.51	±12.91	77.42	±10.00
BMI [kg/m^2]	25.36	±4.27	25.05	±2.73
HR	77.43	±15.00	80.10	±18.60
RR [ms]	801.72	±139.89	779.71	±148.31
PR [ms]	145.61	±21.53	142.68	±32.67
QRS [ms]	92.46	±23.40	95.06	±59.32
QT [ms]	359.01	±69.47	365.39	±33.77
QTc [ms]	421.30	±67.49	397.1	±94.18
P wave [ms]	0.22	±0.38	0.14	±0.15
SV1 [mV]	0.8	±0.47	0.82	±0.35
R wave [mV]	1.38	±0.65	1.38	±0.57
Er [1012/l]	4.26	±0.71	4.69	±0.57
Leuc [109/l]	9.19	±4.80	9.93	±5.76
Hb [g/l]	119.96	±20.91	133.83	±21.29
Hct [%]	0.36	±0.06	0.38	±0.08
MCV [fl]	84.53	±11.86	83.95	±6.73
MCH [pg]	31.19	±24.16	31.45	±10.71
MCHC [g/dl]	329.54	±11.33	328.87	±45.00
Plt [109/l]	273.6	±96.92	270.72	±70.93
Lym [%]	27.01	±11.96	23.74	±9.63
Mo [%]	8.03	±3.60	7.89	±10.05
Gran [%]	65.29	±13.6	66.82	±15.08
RDW [%]	17.60	±2.32	16.3	±10.24

The following CBC parameters were analyzed: red blood cell count (RBC), hemoglobin (Hb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cell distribution width (RDW), platelet count (PLT), mean white blood cell count (WBC), and leukocyte differential count.

One way ANOVA test was performed on ECGs by splitting the participants into four groups:
1) men with cancer;
2) men without cancer;
3) women with cancer;
4) women without cancer.

Multiple comparison test of means was used to obtain the differences between every two groups. Multiple logistic regression analysis was implemented to estimate OR of cancer.

Results and Discussion

Results from ANOVA Analysis

ANOVA analysis of heart rate (HR) was made. The diagram of quartiles of heart rate in males and females is shown on Figure 2. The biggest difference identified was that between the medians in the men with and men without cancer.

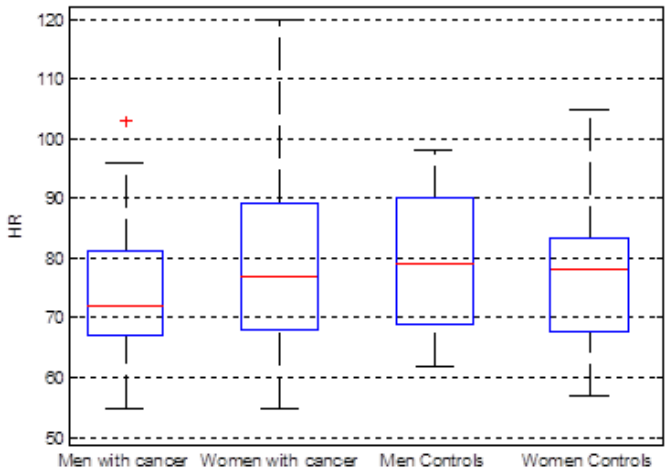

Figure. 1. Diagram of quartiles of HR in male and female groups

Table 3. Data from ANOVA analysis of HR by sex.

Deviations	Sum of squares	Degrees of freedom	Mean value	F – test statistic	p-value
Between groups	1370.877	3.000	456.959	1.789	0.155
Within groups	24006.684	94.000	255.390		
Total	25377.561	97.000			

Table 4. Data from multiple component analysis for HR by sex.

Group one	Group two	Lower bound of CI	Difference between ME	Upper bound of CI
Men with cancer	Men controls	-23.652	-10.378	2.895
Women with cancer	Women controls	-8.135	4.344	16.823

Multiple component analysis of PR interval showed that the differences were not significant and it could be assumed, with a high probability, that there is no connection between PR interval and cancer (Table 5, Table 6)

Table 5. Data from ANOVA analysis of PR by sex

Deviations	Sum of squares	Degrees of freedom	Mean value	F – test statistic	p-value
Between groups	2077.947	3.000	692.647	1.072	0.365
Within groups	60719.247	94.000	645.949		
Total	62797.194	97.000			

Table 6. Data from multiple component analysis for PR interval by sex.

Group one	Group two	Lower bound of CI [ms]	Difference between ME [ms]	Upper bound of CI [ms]
Men with cancer	Men controls	-27.01	-5.90	15.21
Women with cancer	Women controls	-9.59	10.26	30.11

ANOVA analysis of p-wave was made. The multiple component analysis performed showed that the difference between the mean values in the two groups (women with cancer and women without cancer) was 0.137 [ms] with a confidence interval -0.124÷0.397 [ms]. These results confirm that the differences were significant for

women. Consequently, p- wave may be use with an insignificantly degree of probability as a predictor of cancer for women (Table 7, Table 8).

Table 7. Data from ANOVA analysis of p-wave by sex

Deviations	Sum of squares	Degrees of freedom	Mean value	F – test statistic	p-value
Between groups	0.557	3.000	0.186	1.753	0.162
Within groups	9.319	88.000	0.106		
Total	9.876	91.000			

Table 8. Data from multiple component analysis for p-wave by sex

Group one	Group two	Lower bound of CI [ms]	Difference between ME [ms]	Upper bound of CI [ms]
Men with cancer	Men controls	-0.263	0.025	0.314
Women with cancer	Women controls	-0.124	0.137	0.397

ANOVA analysis of QRS complex was made. The value of F-statistic and p- value proved statistically significant differences. The multiple component analysis performed demonstrate that the differences between males and females were bigger than were those between persons with cancer and healthy persons. Results showed that the difference between the mean values in the two groups (men with cancer and men without cancer) was bigger than the difference between the mean values in the two groups (women with cancer and women without cancer) was bigger than. Despite of this, QRS complex may be used as an additional indicator of cancer for men (Table 9, Table 10)

Table 9. Data from ANOVA analysis of QRS by sex

Deviations	Sum of squares	Degrees of freedom	Mean value	F – test statistic	p-value
Between groups	3644.794	3.000	1214.931	2.900	0.039
Within groups	39245.665	94.000	417.507		
Total	42890.459	97.000			

Table 10. Data from multiple component analysis for QRS complex by sex.

Group one	Group two	Lower bound of CI [ms]	Difference between ME [ms]	Upper bound of CI [ms]
Men with cancer	Men controls	-4.673	12.298	29.270
Men with cancer	Women controls	0.036	15.912	31.787
Women with cancer	Women controls	-10.151	5.804	21.759

Is done ANOVA analysis of QT interval after dividing the persons into groups – men with cancer and men without cancer, women with cancer and women without cancer. The values obtained with the multiple component analysis are shown on Table 11 and Table 12. The data showed that after dividing the persons investigated into groups by sex, there was a statistically significant difference between the mathematical expectations for the groups.

Table 11. Data from ANOVA analysis of QT interval by sex.

Deviations	Sum of squares	Degrees of freedom	Mean value	F – test statistic	p-value
Between groups	34885.729	3.000	11628.57	3.430	0.020
Within groups	318671.179	94.000	3390.119		
Total	353556.90	97.000			

Table 12. Data from multiple component analysis for QT interval by sex

Group one	Group two	Lower bound of CI [ms]	Difference between ME [ms]	Upper bound of CI [ms]
Men with cancer	Men controls	-20.260	28.101	76.456
Women with cancer	Women controls	-84.469	-39.004	6.462

ANOVA analysis of QTc was made. There was a difference in the values of medians in groups with cancer and those of individuals without cancer, and this difference was found greater for the male groups. The numerical indices from ANOVA are shown on Table 13.

Table 13. Data from ANOVA analysis of QTc by sex.

Deviations	Sum of squares	Degrees of freedom	Mean value	F – test statistic	p-value
Between groups	58913.617	3.000	19637.872	3.548	0.017
Within groups	520225.249	94.000	5534.311		
Total	579138.866	97.000			

Dividing the groups by sex showed the presence of statistically significant difference between the mathematical expectations for the groups. The results obtained by multiple component analysis are shown on Table 14.

Table 14. Data from multiple component analysis for QTc interval by sex.

Group one	Group two	Lower bound of CI [ms]	Difference between ME [ms]	Upper bound of CI [ms]
Men with cancer	Men controls	12.130	73.920	135.710
Women with cancer	Women controls	-78.053	-19.963	38.128

There was overlapping of quartiles from 25% to 75% for both groups, which showed that the difference between the medians of the two groups was statistically insignificant. The analysis of the results proved that for the group investigated heart rate, RR interval, SV1, and R wave were not a marker for cancer. The QRS complex may be used as a predictor for cancer in the males. The p- wave may be used with an insignificantly degree of probability as a predictor of cancer for women. The most significant ECGs indicators for cancer identified were QT interval and QTc interval. This is why a more extensive research of the ECGs tools for cancer screening is necessary.

Results from Regression Analysis

To assess the combined influence of parameters, logistic regression models with three factors included was performed. The first model included QRS, QT and QTc. This model was presented as follows:

$$\ln\left(\frac{P}{1-P}\right) = b_0 + b_1 * QRS + b_2 * QT + b_3 * QTc$$

where P is the probability for occurrence of cancer and b_0, b_1, b_2, and b_3 are the coefficients of the logistic regression. Coefficients of regression were found for males and females. The p value of overall model fit for women was p=0.4480 and for men was p<0.0009. Results showed that there was statistical significance of model only for men. The values of regression coefficients was b_0=-24.8901, b_1=0.0429, b_2=0.0371 and b_3=-0.0206. On the basis of obtained coefficients it was calculated how the odds ratio (OR) for cancer increased if the respective parameter increased with 5% of mean value. When the QRS increased with 5% of mean value OR for cancer increased 1.22 times. When the QT increased with 5% of mean value OR for cancer increased. 2.01 times. When the QTc increased with 5% of mean value OR for cancer increased 1.55 times.

A threshold of OR is used for assessment of cancer presence among men. The probability of cancer detection (PD) was evaluated as a ratio between the number of men with cancer for which the OR is above threshold and the number of all men with cancer. The probability of false alarm (PFA) that a man without cancer was assessed as a man with cancer was evaluated as a ratio between the number of men without cancer for which the OR is above threshold and the number of all men without cancer. The probabilities of cancer detection and false alarm as functions of threshold are shown on Figure 2.

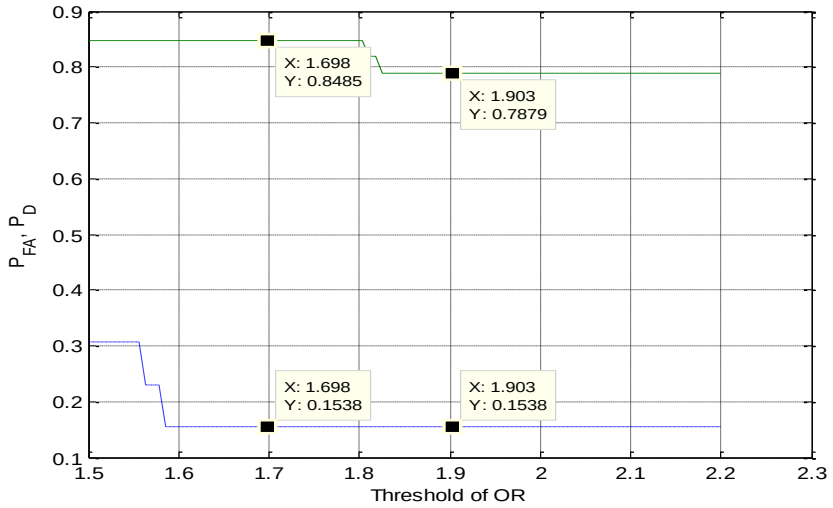

Figure 2. The probabilities of cancer detection and false alarm

If the threshold of OR was chosen 1.7 then the probability of cancer detection was 84.85% and the probability of false alarm was 15.38%. If the threshold of OR was chosen 1.9 then the probability of cancer detection was 78.79% and the probability of false alarm was 15.38%.

Conclusion

The results showed that obtained logistic regression model possessed good abilities for cancer prediction among men, based on the ECGs. Studies proved that further researches for relation between cancer and ECGs will be useful for early cancer screening.

Scientific Ethics Declaration

The author declares that the scientific ethical and legal responsibility of this article published in EPHELS journal belongs to the author.

Acknowledgements or Notes

* This article was presented as an oral presentation at the International Conference on Medical and Health Sciences (www.icmehes.net) held in Antalya/Turkey on November 16-19, 2023.

*Please provide acknowledgements or notes in a separate section at the end of the article before the references.

References

Berson, A.S., Pipberger H.V., (1965), Errors caused by inadequacy of low-frequency response of electrocardiographs. *Digest of 6th International Conference on Biomedical Engineering, Tokyo, Japan, Society of Med Electr Biomed Eng,* (*pp.*13–14). Tokyo*: Okomura Printing Co.*

Daskalov I.K., Dotsinsky I. A., Christov I.I., (1998), Developments in ECG acquisition, preprocessing, parameter measurement and recording. *IEEE Eng in Med and Biol Magazine, 17,*50–58.

Dotsinky, I. (2007). *Review of advanced methods and tools for ECG data analysis*. D.G. Clifford, (F. Azuaje & P. E. McSharry (Eds.),Boston,London:

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Impact of the PK/PD Approach in Therapeutic Drug Monitoring of Gentamicin

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Abstract: Gentamicin, a commonly used hospital aminoglycoside, exhibits a narrow therapeutic index, necessitating careful administration to prevent serious adverse effects. Our study aimed to assess the impact of Therapeutic drug Monitoring (TDM) by integrating Minimum Inhibitory Concentration (MIC) to customize dosage based on bacterial sensitivity. This was prospective study, conducted on a sample of 35 adult patients hospitalized in three university hospital centers in Eastern Algeria. Included patients underwent Therapeutic Drug Monitoring (TDM) of gentamicin based on the determination of Maximum concentration (Cmax), considering the determination of the MIC and achieving a Cmax/CMI ratio ≥ 8 to 10. Pharmaceutical interventions were suggested to clinicians to improve patient care. The mean age of our patients was 51.66 ± 16.72 years. All patients had Cmax values below the therapeutic range. Pathogenic microorganisms were identified in 64% of cases, and only two patients achieved the Cmax/CMI target of 8 to 10 prior to our interventions. In 41% of cases, clinicians heeded our guidance on regular therapeutic monitoring and dose adjustments. The therapeutic target was achieved in 66.6% of cases, with a Cmax/CMI target attainment rate of 44.4%.The use of individualized initial doses of gentamicin, combined with Therapeutic Drug Monitoring based on PK/PD parameters, enhances the chances of therapeutic success and restricts the emergence of bacterial resistance.

Keywords: Gentamicin, Cmax, Cmax/ MIC, PK/PD.

Introduction

Gentamicin, an aminoglycoside commonly prescribed in the hospital for the treatment of severe gram-negative bacterial infections, maintains its role in the management of nosocomial infections due to the possibility of daily single-dose administration. Furthermore, the benefits of combination therapy with a beta-lactam antibiotic are being assessed when dealing with certain gram-negative bacilli (such as Pseudomonas aeruginosa, Enterobacter,

- Selection and peer-review under responsibility of the Organizing Committee of the Conference

Citrobacter, etc.), as well as in the context of treating endocarditis caused by streptococci, enterococci, or methicillin-sensitive Staphylococcus (Padoin et al., 2004).
This is a highly potent and rapidly bactericidal antibiotic, with concentration-dependent killing closely proportional to the maximum serum antibiotic concentration (C_{max}) (Boudia et al., 2023; Mohamed et al., 2012). The therapeutic target ranges from 15 to 25 µg/ml for a daily single dose. In the case of fractionated dosing, the suggested peaks depend on the site of infection. For instance, in Gram-positive infections such as endocarditis, where synergy is sought, gentamicin C_{max} levels should be between 3 and 5 µg/m (Gauzit et al., 2011; Widmer et al., 2008)

However, this medication possesses a narrow therapeutic index and significant inter-individual pharmacokinetic variability (changes in drug blood concentrations based on the patient) as well as intra-individual variability (changes in drug blood concentrations based on variations in physiopathological status) (Bourguignon et al., 2015; Bruno Lacarelle et al., 2007).

As a result, the initially calculated dosage aimed at achieving the desired target at the beginning of treatment may become inadequate after a few days. This can lead to treatment inefficacy and the development of bacterial resistance, as well as potential toxicity, especially nephrotoxicity and ototoxicity, particularly in prolonged treatment (Bourguignon et al., 2010). This renal and auditory toxicity is directly associated with elevated residual concentrations (C_0) (Blunston et al., 2015; Boudia et al., 2023; Hayward et al., 2018).

Therefore, it is advisable to perform frequent monitoring of drug blood concentrations throughout the treatment, to ensure the attainment of pharmacokinetic and pharmacodynamic (PK/PD) targets. This primarily depends on the maximum concentration (C_{max}), which should be eight to ten times higher than the minimum inhibitory concentration (MIC) (C_{max}/MIC $\geq$ 8-10). Furthermore, it is crucial to maintain residual concentrations (C_0) below 1 to 2 µg/l to prevent associated renal and auditory toxicity (Bland et al., 2018; Blunston et al., 2015). Our study aimed to assess the impact of therapeutic drug monitoring of gentamicin (TDM) by incorporating MIC to personalize dosing based on bacterial sensitivity.

Patients and Method

This is a descriptive, prospective study conducted in three university hospital centers in the eastern region of Algeria. The study was carried out on a group of 35 adult patients, hospitalized in various departments of these institutions, who had received gentamicin for a period of at least 5 days. Patient data were extracted from specially designed records, encompassing demographic, clinical information, microbiological data, details on gentamicin dosage, administration methods, as well as the monitoring parameters required for result analysis and interpretation. Informed and voluntary consent was obtained from the patients.

Gentamicin blood samples were collected after reaching a steady-state condition, 48 hours after the first administration. The residual concentration (C_0) was measured at the end of a gentamicin administration interval, approximately 30 minutes before the next dose. Meanwhile, C_{max} was measured 30 to 45 minutes after the completion of the intravenous gentamicin infusion. Gentamicin assay was performed using an enzyme immunoassay (EMIT) method on a Siemens VIVA-E automatic analyzer.

When a severe infection was suspected, specific bacteriological samples were collected based on the type and presumed site of the infection. The objective of these samples was to identify the pathogenic microorganism responsible for the infection, determine its antibiotic sensitivity, and establish the minimum inhibitory concentration (MIC). The liquid dilution method was used to determine MIC values.

Data collection and analysis were carried out using IBM SPSS Statistics version 25. Descriptive analysis results were expressed as frequencies for qualitative variables and as means (± Standard Deviation) for quantitative variables. Bivariate analysis (comparison of qualitative variables) was performed using the Chi-square test. A p-value < 0.05 was considered significant.

Results and Discussion

The 35 patients included in our study were receiving gentamicin in various hospital departments, whether they were medical or surgical in nature. This highlights the relevance of this medication in the management of infections and medical conditions that require its use. The bacteriological profile was documented in 64% of the

patients. The empirical use of gentamicin is based on a thorough examination of the patient's medical history, risk factors, and the potential for bacterial resistance to the antibiotic. During our study, we considered the renal function of our patients by measuring creatinine clearance levels, which were normal in 69.4% of cases. Demographic, clinical, and bacteriological data of the patients are summarized in Table 1.

Table 1. Demographic, clinical, and bacteriological characteristics of the study patients.

Variables		Frequency
Mean Age (years)		51,66 ± 16.72 (18 à 79 years)
Gender		F: 60 %.M: 40 % Sex Ratio: 0.667
Creatinine Clearance (ml/min)		105,42 ± 37,57
Co-morbidity	Arterial Hypertension	33.3 %
	Diabetes	31.0 %
	Dyslipidemia	11.9 %
	Other	23.8 %
Type of infection	Sepsis	45.5 %
	Endocarditis	13.6 %
	Surgical Wound Infection	13.6 %
bacteriological Profile	Empirical	36 %
	Documented	64 %
Isolated Pathogens	Staphylococcus aureus	MIC = 0.5 (30 %) MIC =1.00 (10 %)
	Pseudomonas aeruginosa	MIC = 2 (20 %)
	Klebsiella	MIC = 2 (20 %)
	Escherichia coli	MIC = 1 (10 %)
	Enterobacter	MIC = 1 (10 %)

None of our patients treated with gentamicin received an initial loading dose. Several studies suggest the use of a loading dose based on the patient's ideal body weight to rapidly achieve therapeutic concentrations in the bloodstream and ensure adequate efficacy (Avent et al., 2011; Sous comité de suveillance de l'utilisation des Antibiotiques, 2016; The Gentamicin Improvement Project Group, 2018). In the context of our study, the average daily dose of gentamicin administered was 1.92 ± 0.75 mg/kg via single daily intravenous infusion in 57% of cases, and was fractionated into two administrations per day in 5.7% of cases.

These doses are lower than those reported in other studies, such as the study conducted by Claire Roger and her colleagues in France in 2015, which included 24 patients treated with gentamicin. The average initial dose of gentamicin was 6.6 ± 2.3 mg/kg with a median of 5.9 mg/kg. None of the patients received a dose lower than 3 mg/kg (Roger et al., 2015). It has been demonstrated that a daily single dose of gentamicine achieves PK/PD targets for numerous bacterial strains and enhances tissue penetration due to higher plasma/tissue concentration gradients (Hansen et al., 2001; Kovačević et al., 2016). However, for the treatment of endocarditis, fractionated dosing is preferred, with the daily dosage typically divided into 2 to 3 injections per day, every 8 or 12 hours (Gauzit et al., 2011).

The residual concentrations C_0 were measured for the 35 patients in our study, and it was found that 82.9% of the patients had C0 levels below 1 µg/ml, and these levels were correlated with the daily doses administered (p-value = 0.040). Our results are consistent with those of Kovacevic T. et al, conducted in 2016. The C0 concentrations of 25 out of 31 patients (80.6%) were within the therapeutic range (Kovačević et al., 2016). Among the 31 patients treated with gentamicin, the determination of C_{max} revealed under-dosing, with levels below 15 µg/ml, averaging 5.28 ± 2.74 µg/ml.

In the 4 cases where gentamicin was used to achieve synergy in the treatment of infective endocarditis, 3 cases, or 75%, had a C_{max} within the therapeutic range (3 - 5 µg/ml), while only one case had a Cmax exceeding 5 µg/ml, indicating an overdose. In the study by Kovacevic T. et al., conducted in 2016, 80.6% of C_{max} values were within the therapeutic range established at 8-10 mg/ml (Kovačević et al., 2016). This disparity can be attributed to the administration of significantly lower doses in our patient population (p-value = 0.001).

In our study, only two cases achieved the target C_{max}/CMI ratio of 8 to 10 prior to our interventions. In contrast, the study conducted by Coste A. on 49 patients treated with gentamicin at the university hospital of Nantes in France in 2019, demonstrated that all patients had a C_{max}/CMI ratio greater than or equal to 8 (Coste et al., 2020) Following the results of gentamicin assays and after analyzing the physiopathological characteristics of each patient, their biological and bacteriological parameters, pharmaceutical issues in gentamicin prescription were identified. In 32% of cases, dosages were sub-therapeutic, requiring dosage adjustments and regular therapeutic monitoring.

In 41% of cases, clinicians followed our recommendations. Dosage adjustments were made for 8 patients, and the therapeutic target was achieved in 66.6% of cases, with an increase in daily doses administered (average of 195 ± 72.31 mg). These dosages were closely correlated with patient weight and clearance (p-value of 0.05 and 0.014, respectively). After each dose adjustment, new blood samples were taken 48 hours later. C_{max} levels entered the therapeutic range in 62.5% of cases, with an increase in the average to 9.55 ± 8.74 mg (Figure1).

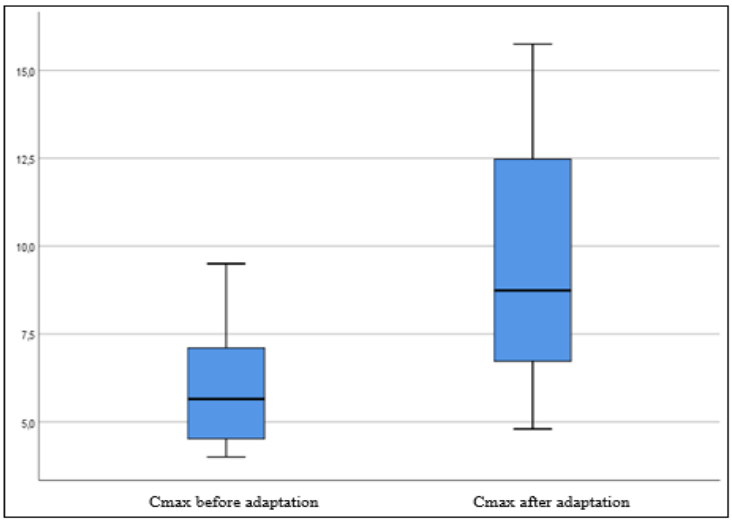

Figure 1. Schéma de la différence de médianes des Cmax avant et aprés adaptation posologique

The specific objective of a C_{max}/CMI ratio between 8 and 10 was achieved in 44.4% of cases, showing a significant correlation with the patients' creatinine clearance (p-value = 0.047).

Conclusion

The use of individualized initial doses of gentamicin, combined with therapeutic drug monitoring based on pharmacokinetic/pharmacodynamic (PK/PD) parameters, enhances the chances of therapeutic success and limits the emergence of bacterial resistance. Our study revealed a significant rate of underdosing due to sub-therapeutic initial doses and inadequate administration methods. All these factors expose patients to therapeutic failure or an increased duration of hospitalization.

The implementation of therapeutic drug monitoring based on the PK/PD approach represents a significant advancement and provides the rational basis for improving the selection of appropriate gentamicin doses and their administration methods based on the bacteria involved and the patient's pharmacokinetic characteristics, with a promising prospect of enhancing the management of severe patients."

Recommendations

For optimal use of gentamicin, it is imperative to select an individualized dose based on body weight and renal function. Furthermore, it is essential to regularly monitor gentamicin blood concentrations. The use of PK/PD principles should guide the selection of doses and administration methods based on the bacteria involved and patient characteristics, especially for serious infections. It is also crucial to optimize the route and timing of administration, taking into account the patient's condition and the site of infection. Finally, close collaboration with pharmacists is necessary to ensure compliance with dosing guidelines, appropriate monitoring, and personalized dose adjustments.

Scientific Ethics Declaration

The authors declare that the scientific ethical and legal responsibility of this article published in EPHELS journal belongs to the authors.

Acknowledgements or Notes

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References

Avent, M. L., Rogers, B. A., Cheng, A. C., & Paterson, D. L. (2011). Current use of aminoglycosides : Indications, pharmacokinetics and monitoring for toxicity. *Internal Medicine Journal*, *41*(6), 441-449.

Bland, C. M., Pai, M. P., & Lodise, T. P. (2018). Reappraisal of contemporary pharmacokinetic and pharmacodynamic principles for ınforming aminoglycoside dosing. *Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy*, *38*(12), 1229-1238.

Blunston, M. A., Yonovitz, A., Woodahl, E. L., & Smolensky, M. H. (2015). Gentamicin-induced ototoxicity and nephrotoxicity vary with circadian time of treatment and entail separate mechanisms. *Chronobiology International*, *32*(9), 1223-1232.

Boudia, F., Fetati, H., Mekaouche, F. Z. N., Memou, A., Kaidari, H., Larabi, A., Mansouri, I., Djoudad, K., Meknassi, D., Zerdoumi, F., & Toumi, H. (2023). Gentamicine en hémodialyse : Quelles pratiques en milieu hospitalier? *Algerian Journal of Health Sciences*, *5*(1), 1-10.

Bourguignon, L., Cazaubon, Y., Goutelle, S., & Guitton, J. (2015). *Suivi thérapeutique pharmacologique des aminosides : Amikacine, gentamicine, tobramycine.* Retrieved from https://www.em, consulte.com

Bourguignon, L., Goutelle, S., Burdin de Saint Martin, J., Guillermet, A., Bouniot, P., Maire, P., & Ducher, M. (2010). Variabilité pharmacocinétique intra-individuelle et traitement antibiotique prolongé. *Médecine et Maladies Infectieuses*, *40*(1), 38-41.

Coste, A., Deslandes, G., Jalin, L., Corvec, S., Caillon, J., Boutoille, D., Grégoire, M., & Bretonnière, C. (2020). PK/PD targets of amikacin and gentamicin in ICU patients. *Medecine Et Maladies Infectieuses*, *50*(8), 709-714.

Gauzit, R., Cohen, R., & Chidiac, C. (2011). Mise au point sur le bon usage des aminosides administres par voie injectable. *AFSSAPS.* Retrieved from https://www.infectiologie.com

Hansen, M., Christrup, L. L., Jarløv, J. O., Kampmann, J. P., & Bonde, J. (2001). Gentamicin dosing in critically ill patients. *Acta Anaesthesiologica Scandinavica*, *45*(6), 734-740.

Hayward, R. S., Harding, J., Molloy, R., Land, L., Longcroft-Neal, K., Moore, D., & Ross, J. D. C. (2018). Adverse effects of a single dose of gentamicin in adults : A systematic review. *British Journal of Clinical Pharmacology*, *84*(2), 223-238.

Kovačević, T., Avram, S., Milaković, D., Špirić, N., & Kovačević, P. (2016). Therapeutic monitoring of amikacin and gentamicin in critically and noncritically ill patients. *Journal of Basic and Clinical Pharmacy*, *7*(3), 65-69.

Lacarelle, B., Baltasat, A., Bouquet, S., & Venisse, N. (2007). Suivi thérapeutique pharmacologique de la gentamicine. *EM-Consulte*. Retrieved from https://www.em-consulte.com/article/61338/suivi-therapeutique-pharmacologique-de-la-gentamic

Mohamed, A. F., Nielsen, E. I., Cars, O., & Friberg, L. E. (2012). Pharmacokinetic-pharmacodynamic model for gentamicin and ıts adaptive resistance with predictions of dosing schedules in newborn ınfants. *Antimicrobial Agents and Chemotherapy*, *56*(1), 179-188.

Padoin, C., Jacolot, A., Nicolas, P., Soreda, S., & Petitjean, O. (2004). Apport du dosage plasmatique des aminoglycosides. *Revue Française des Laboratoires*, *2004*(365), 27-34.

Roger, C., Nucci, B., Molinari, N., Bastide, S., Saissi, G., Pradel, G., Barbar, S., Aubert, C., Lloret, S., Elotmani, L., Polge, A., Lefrant, J.-Y., Roberts, J. A., & Muller, L. (2015). Standard dosing of amikacin and gentamicin in critically ill patients results in variable and subtherapeutic concentrations. *International Journal of Antimicrobial Agents*, *46*(1), 21-27.

Sous comité de suveillance de l'utilisation des Antibiotiques. (2016). *Guıde d'utılısatıon de la gentamıcıne et de la tobramycıne chez l'adulte*. Chu De Quebec.Unıversıte De Laval.

The Gentamicin Improvement Project Group. (2018). *Gentamicin : Guidelines for once daily usage in adult and pediatric settings*. Royal College of Physicians of Irland.

Widmer, N., Werner, D., Grouzmann, E.,, & Eap, C. B. (2008). Suivi thérapeutique des médicaments (II) La pratique clinique. *Revue Médicale Suisse*, *4*, 1649-1660.

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Stability of Amino Acids in Stored Dried Blood Spots: Retrospective Analysis

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Abstract: Residual newborn screening dried blood spots (DBS) are a valuable resource for research in the retrospective diagnosis of inborn errors of metabolism and biomarker analysis. Many metabolites are subject to degradation depending on time and storage conditions, such as temperature and humidity. We studied the stability of 15 amino acids (AAs) in dried blood spots stored in a refrigerator after newborn screening. Methods: We retrospectively analyzed the levels of 15 AAs in 248 residual DBS from the Kazakhstan neonatal screening program using tandem mass spectrometry. DBS were stored at 4°C and 55-70% humidity and randomly selected during 2019-2022. Amino acid stability was assessed using linear regression and estimating the decrease in concentration of each metabolite during each year. Results: Retrospective analysis of dried blood spot samples stored for one to four years showed that the decrease in concentrations of 15 AA occurred in order from most stable to least stable: valine, proline, isoleucine, leucine, tyrosine, phenylalanine, alanine, arginine, hydroxyproline, methionine, citrulline, glutamate, ornithine, glycine, and glutamine. Alanine, arginine, hydroxyproline, glycine, and glutamate decayed in line with linear regression. Conclusion: Storing dried blood spots at 4°C and 55-70% humidity is not optimal for amino acid stability. Data obtained from measuring amino acid levels in dried blood spots stored over time should be corrected to reduce the incidence of negative and false positive results.

Keywords: Amino acids, Dried blood spot, Metabolite stability, Inborn errors of metabolism, Tandem mass spectrometryю

Introduction

Dried blood spots (DBS) allow for quick and easy collection and storage of human biospecimens (Freeman et al., 2018; Ward et al., 2021). DBS collection has many advantages over “standard” venous blood collection (Trifonova et al., 2019). DBS cards collected for newborn screening (NBS) are often stored for an extended period (Strnadova et al., 2007). DBS samples are collected from almost all of the more than 400 thousand children born in Kazakhstan annually. Unused parts of these samples (residual samples) are stored for three years after completion of testing.

Residual newborn screening DBS samples are a valuable resource for research (Benkendorf et al.,2010; Rothwell et al.,2019). Stored DBS can provide valuable samples for retrospective diagnosis of inborn errors of metabolism (IEM) and biomarker analysis, as well as for validation of NBS programs (Strnadová et al., 2007; Freeman et al., 2018; van Rijt et al., 2020; Dijkstra et al., 2020). 2023). This is especially important in resource-constrained countries (Ward et al., 2021; Ottosson et al., 2023).

However, diagnostic uncertainties arising from the instability of metabolites during long-term storage have not been systematically and comprehensively studied (Strnadová et al., 2007; Fingerhut et al., 2009; van Rijt et al., 2020; Shimada et al., 2022; Dijkstra et al.,2023; Ottosson et al.,2023). When considering using DBS samples for clinical or research purposes, the data obtained from secondary use should be interpreted carefully (Murphy et al., 2018). Retrospective analyses using stored DBS are limited by the lack of information on the long-term stability of analytes in stored samples (Shimada et al., 2022).

Many metabolites are subject to time-dependent degradation. The stability of metabolites is assessed by the change in their concentration in DBS over time (van Rijt et al., 2020; Dijkstra et al., 2023). The stability of metabolites in dried blood spots must be assessed over the relatively short time intervals required to transport the sample to the laboratory as over the long period of sample storage with the possibility of re-analysis.

Factors influencing the stability of metabolites include storage time, temperature, and humidity. Humidity can cause or exacerbate analyte degradation, mainly due to hydrolysis reactions (Wagner et al., 2016). DBS requires low humidity and low-temperature conditions for transportation and storage (Adam et al., 2011; Golbahar et al., 2014). Adam et al. (2011) believe that most of the degradation of metabolic markers is due to the adverse effects of storage at high humidity and temperatures of 37°C. Among other things, it should be remembered that the quality of blood parameters analyzed from DBS samples depends on field conditions, especially the spot size. Smaller DBSs are associated with lower measured metabolite levels (Moat et al., 2020; Crimmins et al., 2020; Börsch-Supan et al., 2021; Groh et al., 2022).

According to Ottosson et al. (2023), the metabolomics of DBS samples during long-term storage at -20 °C in biobanks is suitable for retrospective epidemiological studies. Palmer et al. (2019) recommend collecting and transporting DBS samples within 28 days at room temperature and storing for more extended periods at -20 or -80 °C.

Michopoulos et al. (2011) suggest that the stability of metabolites in DBS samples is reduced if the cards are not stored at a temperature of at least -20°C, and preferably -80°C. In addition, untreated cards are recommended to minimize background interference incurred. Thus, factors influencing the stability of metabolites in DBS samples include storage time, temperature, humidity, and the type of paper used to collect DBS samples (Michopoulos et al., 2011; Wagner et al., 2016). Assessing annual metabolite decline may allow retrospective diagnosis of IEM in DBS stored over long periods (Strnadová et al., 2007). Van Rijt et al. (2020) recommend including follow-up DBS in diagnostic retrospective cohorts and validation studies using fresh samples and repeatedly reestimating cut-off values.

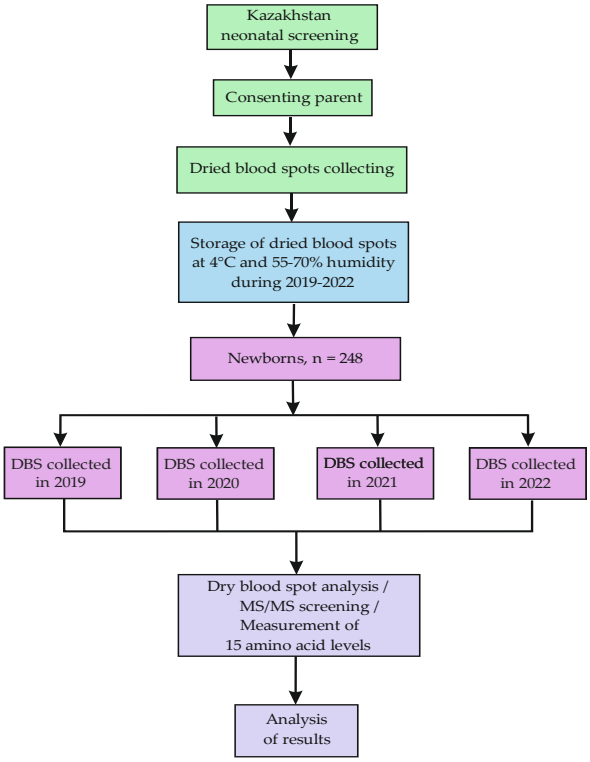

Figure 1. Flowchart of retrospective analysis of dried blood spots.

Objectives

The study aims to determine the stability of 15 amino acids in DBS stored in a refrigerator after NBS.

Methods

Data Sources

The data used in our analysis were obtained by studying the level of 15 AAs in 248 DBS obtained from healthy newborns aged 1-3 days during the Kazakhstan neonatal screening (Figure 1). The DBS stored in the archives were randomly selected. DBS stored at 4°C and 55-70% humidity were randomly selected during 2019-2022 (Table 1).

Table 1. Total storage time and storage conditions of DBS employed in this study.

Group	2019	2020	2021	2022	2023	Storage Time (yrs)
2019	Collection and storage at +4◦ C				Transport to the	3.5-4.5
2020		Collection and storage at +4◦ C			laboratory +	2.5-3.5
2021			Collection and storage at +4◦ C		storage at +4◦ C	1.5-2.5
2022				Collection and storage at +4◦ C	AA measurements	0.5-1.5

In Kazakhstan, neonatal screening is carried out nowadays for two hereditary diseases - phenylketonuria and congenital hypothyroidism, the most represented screening diseases in most countries (Therrell et al., 2018). However, the MS/MS tandem mass spectrometry method is not used in the state newborn screening program, and ENBS for AAD, OA, and FAOD is not carried out in Kazakhstan. Blood samples from newborns on Guthrie cards were previously (until 2019) stored for one year in a refrigerator at 4°C, then at room temperature. Since 2019, DBS samples have been stored permanently at 4°C. The study was approved by the Bioethics Committee of the West Kazakhstan Marat Ospanov Medical University (Ref. No. 7, 09/09/2020.) Written informed consent (IC) was obtained from the parents and/or legal guardians of children after birth to collect a DBS sample.

Mass Spectrometry Analysis

The Neobase2 TM Non-derivatized MSMS kit (PerkinElmer, Wallac Oy, Turku, Finland) were used to quantify 15 amino acids in DBS according to the manufacturer's instructions. Vial with lyophilized isotope-labeled internal standards (IS) containing 2H3-Alanine (Ala IS), 2H4, 13C-Arginine (Arg IS), 2H2-Citrulline (Cit IS), 13C5-Glutamine (Gln IS), 13C5-Glutamic acid (Glu IS), 15N,2-13C-Glycine (Gly IS), 2H3-Leucine (Leu IS), 2H3-Isoleucine (Leu IS), 2H3-Hydroxyproline (Leu IS), 2H3-Methionine (Met IS), 2H6-Ornithine (Orn IS), 13C6-Phenylalanine (Phe IS), 13C5-Proline (Pro IS), 13C6-Tyrosine (Tyr IS), 15N-13C5-Valine (Val IS) was being recovered by adding 1.4 ml of the extraction solution that has been included in the Neobase 2 kit. The Extraction Working Solution (EWS) IS was prepared by diluting the recovered internal standards with the extraction solution of 1:100 (v/v).

DBS were analyzed using a Shimadzu LCMS-8050 Triple Quadrupole Mass Spectrometer (Shimadzu Corporation, Kyoto, Japan). Sample preparation was based on extraction followed by derivatization into oil esters. Level I and Level II (low standard and high standard) dried blood drops were included with each assay lot of the Neobase2 TM Non-derivatized MSMS kit to monitor system accuracy and precision.

To analyze amino acids and acylcarnitines, stored DBS card samples were brought to room temperature (+18 to +25°C) before extraction. A 3.2 mm disc (equivalent to ~3.1 µl of whole blood) was punched out of one dried blood spot with a diameter of 3.2 mm using a Wallac DBS Puncher (PerkinElmer, Wallac Oy, Mustionkatu 6, FI-20750 Turku, Finland) into the well of the 96-well polystyrene U-bottom microplate supplied with the Neobase2 TM Non-derivatized MSMS kit. After adding 125 µL of working extraction solution to each well of the microplate, the plate was covered with an adhesive aluminum film and incubated for 30 minutes at room temperature on a microplate shaker with a shaking speed of 650 rpm. After incubation, 100 µL of the supernatant

was transferred to a new 96-well U-bottom microplate, covered with aluminum foil to reduce evaporation, and incubated for 1 hour. The plate was then placed into the Shimadzu LCMS-8050 Triple Quadrupole Mass Spectrometer autosampler, and 5 µL of supernatant was injected into the LCMS for analysis.

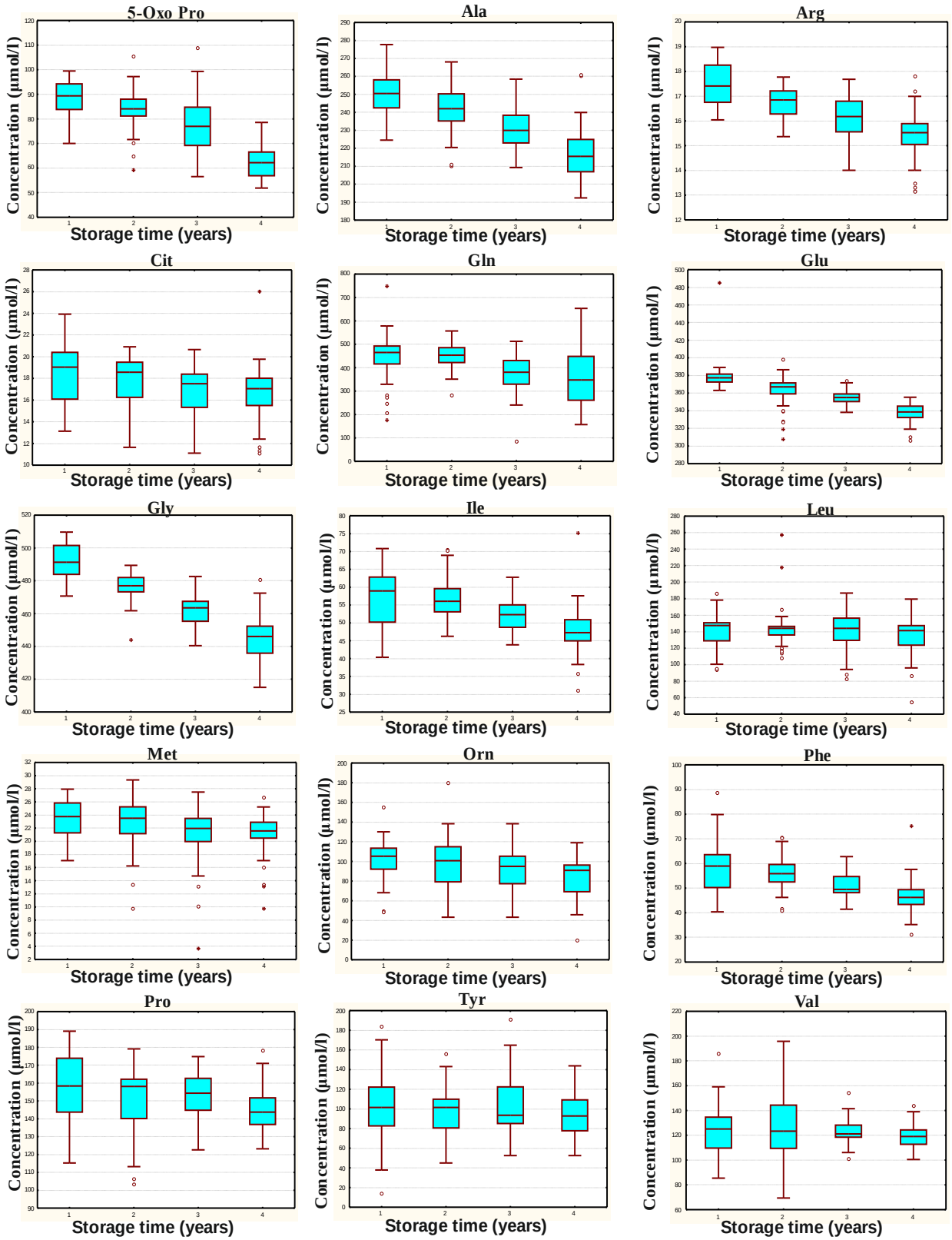

Figure 2. Changes in the concentration of amino acids in dried blood spots at a temperature of 4°C with humidity 55-70% during storage for four years.
(The boxplots represent 1st quartile, median, and 3rd quartile. The whiskers extend to the non-outlier range. Circles = non-extreme outliers, asterisks = extreme outliers).

Statistical Analysis

Shapiro-Wilk and Kolmogorov-Smirnov tests were used to check the normality of the distribution. The data obtained in the study demonstrated that the distribution of amino acids in DBS differs from normal. Me (median) and quartiles (IQR, interquartile range) were used for descriptive statistics of the samples. Nonparametric tests (Kruskal-Wallis H-test) were used to check differences between AA concentrations depending on storage time.Amino acid stability was assessed using linear regression and estimating the decrease in concentration of each metabolite during each year. Two-sided levels <0.05 are assumed to be statistically significant. Statistical analysis was carried out using the statistical packages IBM SPSS v. 23.0 (IBM, Armonk, NY, USA) and Statistica (StatSoft, Inc., Tulsa, OK, USA, v. 10).

Results and Discussion

The study results of the amino acids stability in DBS stored for one to four years in a refrigerator at a temperature of 4°C and 55-70% humidity are presented in a series of box plots in Fig. 2, and linear diagrams of amino acid degradation in Fig. 3.

Retrospective analysis of DBS samples stored for one to four years showed that the decrease in concentrations of 15 AA occurred in the order from most stable to least stable: valine, proline, isoleucine, leucine, tyrosine, phenylalanine, alanine, arginine, hydroxyproline, methionine, citrulline, glutamate, ornithine, glycine, and glutamine.

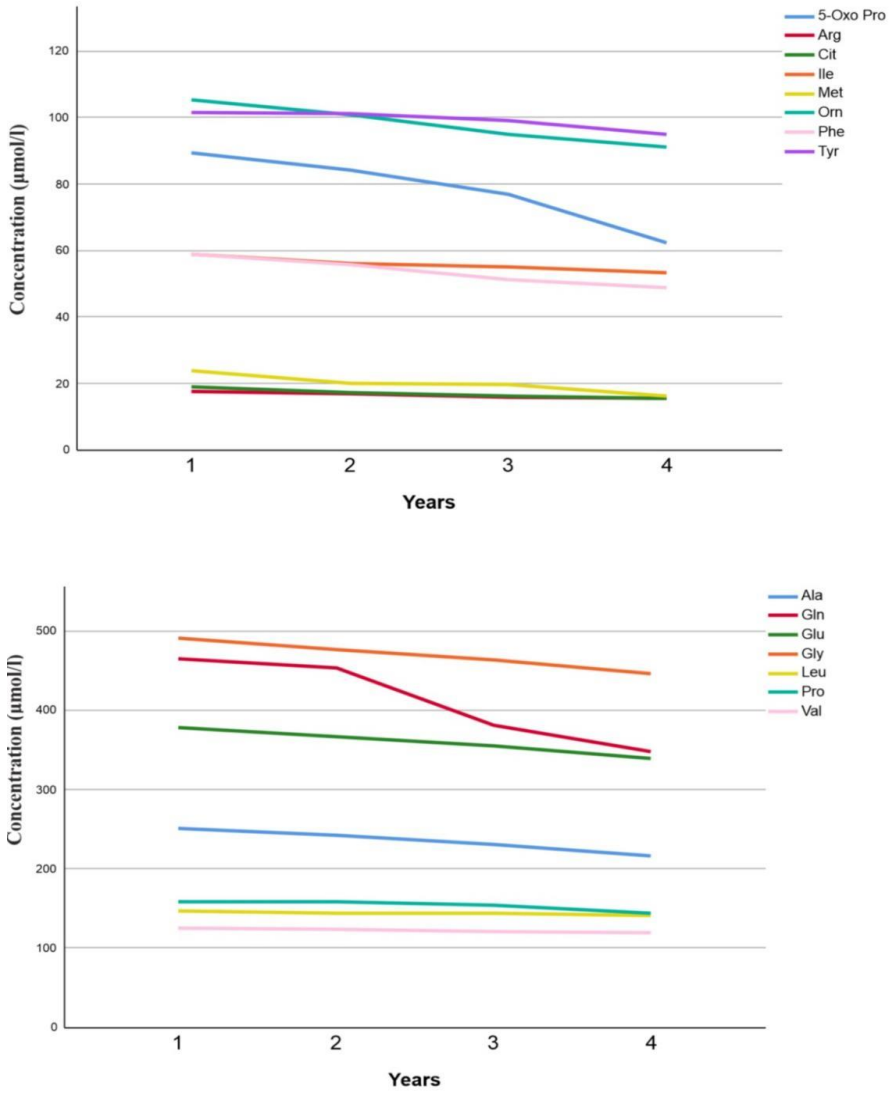

Figure 3. Degradation of amino acids stored at 4°C and humidity 55-70% within four years.

The results of linear regression analysis, where the concentrations of amino acids in DBS were considered dependent variables and the storage time of samples was considered independent variables, are presented in Table 3. Alanine, arginine, hydroxyproline, glycine, and glutamate decayed in line with linear regression.

Table 3. The linear regression formulae and coefficient correlation of R^2

Amino acid, µmol/l	R^2	linear regression formulae	Durbin-Watson statistic
5-Oxo Pro	0.585	y =-8,80x + 99.86	1.28
Ala	0.504	y =-11.08x + 252.52	1.73
Arg	0.549	y =-0.76x + 18.25	1.69
Cit	0.214	y =-0.96x + 19.03	1.91
Gln	0.163	y =-34.94x + 496.91	1.87
Glu	0.573	y =-13.04x + 391.14	2.07
Gly	0.745	y =-16.08x + 508.95	1.85
Ile	0.075	y =-1.29x + 58.79	1.81
Leu	0.004	y =-1.18x + 144.20	1.81
Met	0.430	y =-2.26x + 25.49	1.98
Orn	0.096	y =-6.33x + 109.99	1.95
Phe	0.217	y =-2.97x + 61.21	1.85
Pro	0.058	y =-3.38x + 159.52	2.01
Tyr	0.012	y =-2.21x + 104.82	1.67
Val	0.008	y =-1.34x + 125.87	2.08

*The shaded rows indicate the items maintaining a linear correlation with $R^2 \geq 0.5$.

This study distinguishes three conditional groups of amino acids: those demonstrating the most excellent stability under storage conditions at a temperature of +4°C and a humidity of 55-70%, amino acids with high degradation rates, and a group of amino acids with intermediate rates. The first group included valine, proline, isoleucine, and leucine; the intermediate group had tyrosine, phenylalanine, alanine, arginine, hydroxyproline, and the most unstable were methionine, citrulline, glutamate, ornithine, glycine, and glutamine. According to various studies, the most significant factors influencing the stability of amino acids in DBS are storage time, temperature, and humidity.

Different DBS storage conditions hamper comparative analysis of studies describing amino acid stability in DBS. Some studies describe the stability of the metabolome in DBS under long-term storage conditions. Thus, Prentice et al. (2013) evaluated the stability of metabolites in DBS stored at various temperatures (+21, -20, and -80°C) for two years. Shimada et al. (2022) studied the characteristic stability profiles of amino acids in DBS stored in a refrigerator at 5° C after newborn screening at 1, 3, 6 months, one, and two years of storage. Ottosson et al. (2023) found that most (71%) of the metabolome of neonatal DBS was stable for ten years of storage at -20° C. Dijkstra et al. (2023) investigated the five-year stability of 23 amino acids in residual DBS stored for five years (one year at +4° C and four years at room temperature). Some authors described the stability of amino acids under short-term storage conditions. Ward et al. (2021) found that more than 80% of metabolites are chemically stable in DBS stored at room temperature for a week. Golbahar et al. (2014) examined the short-term effect of heat and humidity on the levels of 7 amino acids for eight days. Han et al. (2018) showed that most amino acids are stable in DBS samples during 4 hours of sunlight exposure.

The relative stability of proline (Shimada et al., 2022, Dijkstra et al., 2023), isoleucine (Dijkstra et al., 2023), alanine (Shimada et al., 2022, Dijkstra et al., 2023), phenylalanine (Shimada et al., 2022, Dijkstra et al., 2023), and valine (Dijkstra et al., 2023) are confirmed by the results of this study. Research data from Han et al. (2018), Moat et al. (2020), and Dijkstra et al. (2023) confirmed the instability of methionine, both under short-term and long-term storage conditions. Methionine is one of the most unstable amino acids in DBS, and at room temperature, it is degraded by at least 50% in six months (Han et al. 2018). Rapidly degrading amino acids also include citrulline, glycine, ornithine (Strnadová et al., 2007; Dijkstra et al., 2023), and glutamine (Dijkstra et al., 2023). In our study, glutamate also showed a high level of degradation.

Conclusion

Storing dried blood spots at +4°C and 55-70% humidity is not optimal to ensure the stability of amino acids. Some of the AA in DBS from neonatal screening stored under these conditions can have undergone significant

degradation. This may lead to misinterpretation of test results for retrospective biomarker studies and IEM diagnosis.

Recommendations

Data obtained from measuring amino acid levels in DBS stored for a specific time should be adjusted to reduce the incidence of negative and false positive results. Conducting retrospective analyses from DBS samples requires the development of reference values established for similarly stored blood samples from DBS of healthy newborns.

Scientific Ethics Declaration

The authors declare that the scientific ethical and legal responsibility of this article published in EPHELS journal belongs to the authors.

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References

Adam, B. W., Hall, E. M., Sternberg, M., Lim, T. H., Flores, S. R., O'Brien, S., Simms, D., Li, L. X., De Jesus, V. R., & Hannon, W. H. (2011). The stability of markers in dried-blood spots for recommended newborn screening disorders in the United States. *Clinical biochemistry*, *44*(17-18), 1445–1450.

Benkendorf, J., Goodspeed, T., & Watson, M. S. (2010). Newborn screening residual dried blood spot use for newborn screening quality improvement. *Genetics in Medicine: Official Journal of the American College of Medical Genetics*, *12*(12), 269–272.

Dijkstra, A. M., de Blaauw, P., van Rijt, W. J., Renting, H., Maatman, R. G. H. J., van Spronsen, F. J., Maase, R. E., Schielen, P. C. J. I., Derks, T. G. J., & Heiner-Fokkema, M. R. (2023). Important lessons on long-term stability of amino acids in stored dried blood spots. *International Journal of Neonatal Screening*, *9*(3), 34.

Fingerhut, R., Ensenauer, R., Röschinger, W., Arnecke, R., Olgemöller, B., & Roscher, A. A. (2009). Stability of acylcarnitines and free carnitine in dried blood samples: implications for retrospective diagnosis of inborn errors of metabolism and neonatal screening for carnitine transporter deficiency. *Analytical Chemistry*, *81*(9), 3571–3575.

Freeman, J. D., Rosman, L. M., Ratcliff, J. D., Strickland, P. T., Graham, D. R., & Silbergeld, E. K. (2018). State of the Science in Dried Blood Spots. *Clinical Chemistry*, *64*(4), 656–679.

Golbahar, J., Altayab, D. D., & Carreon, E. (2014). Short-term stability of amino acids and acylcarnitines in the dried blood spots used to screen newborns for metabolic disorders. *Journal of Medical Screening*, *21*(1), 5–9.

Murphy, M. S. Q., Hawken, S., Cheng, W., Wilson, L. A., Lamoureux, M., Henderson, M., Potter, B., Little, J., Chakraborty, P., & Wilson, K. (2018). Metabolic profiles derived from residual blood spot samples: A longitudinal analysis. *Gates Open Research*, *2*, 28.

Ottosson, F., Russo, F., Abrahamsson, A., MacSween, N., Courraud, J., Nielsen, Z. K., Hougaard, D. M., Cohen, A. S., & Ernst, M. (2023). Effects of long-term storage on the biobanked neonatal dried blood spot metabolome. *Journal of the American Society for Mass Spectrometry*, *34*(4), 685–694.

Rothwell, E., Johnson, E., Riches, N., & Botkin, J. R. (2019). Secondary research uses of residual newborn screening dried bloodspots: a scoping review. *Genetics in Medicine: Official journal of the American College of Medical Genetics*, *21*(7), 1469–1475.

Shimada, Y., Kawano, N., Goto, M., Watanabe, H., & Ihara, K. (2022). Stability of amino acids, free and acyl-carnitine in stored dried blood spots. *Pediatrics International*, *64*(1), e15072.

Strnadová, K. A., Holub, M., Mühl, A., Heinze, G., Ratschmann, R., Mascher, H., Stockler-Ipsiroglu, S., Waldhauser, F., Votava, F., Lebl, J., & Bodamer, O. A. (2007). Long-term stability of amino acids and acylcarnitines in dried blood spots. *Clinical Chemistry*, *53*(4), 717–722.

Trifonova, O. P., Maslov, D. L., Balashova, E. E., & Lokhov, P. G. (2019). Evaluation of dried blood spot sampling for clinical metabolomics: effects of different papers and sample storage stability. *Metabolites*, *9*(11), 277.

van Rijt, W. J., Schielen, P. C. J. I., Özer, Y., Bijsterveld, K., van der Sluijs, F. H., Derks, T. G. J., & Heiner-Fokkema, M. R. (2020). Instability of acylcarnitines in stored dried blood spots: The impact on retrospective analysis of biomarkers for inborn errors of metabolism. *International Journal of Neonatal Screening*, *6*(4), 83.

Wagner, M., Tonoli, D., Varesio, E., & Hopfgartner, G. (2016). The use of mass spectrometry to analyze dried blood spots. *Mass Spectrometry Reviews*, *35*(3), 361–438.

Ward, C., Nallamshetty, S., Watrous, J. D., Acres, E., Long, T., Mathews, I. T., Sharma, S., Cheng, S., Imam, F., & Jain, M. (2021). Nontargeted mass spectrometry of dried blood spots for interrogation of the human circulating metabolome. *Journal of Mass Spectrometry: JMS*, *56*(8), e4772.

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The Urgency of Anti-Sexual Violence Education for Higher Education Students

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Abstract: Sexual violence constitutes a crime against humanity in numerous nations, including Indonesia. Numerous incidents of sexual violence have occurred in the realm of education, particularly in higher education, which should be a safe environment for students to study. The purpose of this study is to delve deeper into the urgency of anti-sexual violence education in higher education. The method employed in this study was a qualitative method with a literature study approach. The results of this study showed that anti-sexual violence education in higher education is very important because there are many cases of sexual violence in Indonesia which involve various components in higher education. The forms of anti-sexual violence education efforts canbe carried out by developing anti-sexual violence learning models in higher education and through various programs that encourage the active involvement of students in efforts to support anti-sexual violence. The researchers of this study concluded that education on anti-sexual violence for students in higher education is highly important and can be implemented in a variety of ways to strengthen students' knowledge and involvement in dealing with various cases of sexual violence.

Keywords: Anti-sexual violence, Sexual education, Sexual health

Introduction

Sexual violence is a very concerning phenomenon. This relates to crimes against humanity and cannot be tolerated. All parties must take responsibility for this issue, especially in fostering the character of anti-sexual violence through education. Various educational facilities must accommodate students' sense of security from sexual violence. Higher education is a place that should be free from sexual violence and even produce students who are ready to participate in efforts to instil anti-sexual violence in society and know what to do when they see sexual violence occurring in their environment.

Sexual violence is not just an ordinary act of violence. Sexual violence is included in acts of violence against humans, namely crimes against humanity. This crime is included in the form of The Most Serious Crime, which also shows the role of international law against this crime. The problem of sexual violence thatdoes not only exist in Indonesia is an extraordinary danger for the international world. The ongoing international conflict is also a place for international sexual violence to continue. Women and children are vulnerable, as can be seen from data from The International Rescue Committee, namely that 40% of 190 women and children in the Dara'a and Quneitra areas became victims of sexual violence even when accessing humanitarian assistance from

International Organization personnel. (Hilmi & Airlangga, 2019).

Crimes regarding sexual violence have continued to increase every year since 2014. Efforts to overcome and suppress this is through educational channels, especially effective educational media, to reduce the number of cases of sexual violence (Arsyati et al., 2017 & Damayanti, 2017). Sexual violence is not only an international problem. Sexual violence is also a problem in the regional realm within the territory of the unitary state of the Republic of Indonesia, including the city of Cirebon. Cirebon City is a city designated as an emergency city for sexual violence. This can be seen from the data on the increase in the number of sexual violence from 2016. There were thirty cases of sexual violence in 2016 that occurred in the city of Cirebon. Cases continued to increase until, in 2017, there were one hundred twenty-six cases of sexual violence, and they rose again to one hundred forty-seven cases of sexual violencein 2018. (Rahman & Sarip, 2021)

The education sector, which is supposed to be a safe haven, has become a place for sexualviolenceto occur. This is not only happening in Indonesia but also in various countries. The movement against. Sexual violence was born in the field of education. Students are expected to have respect, but the many cases of sexual violence on campus have led to many movements fighting for justice for various victims of sexual violence. Through various campaigns involving social media and technology. (Zeng, 2020)

Students, as adult citizens, tend to be more involved with facilitated universities. This is in line with the concept of citizen engagement (Flanagan & Levine 2010) that young citizens, such as students with college experience, show far more involvement as citizens than those who are not in college. Ideally, tertiary institutions directly strengthen citizen skills and citizen knowledge with various lessons, especially civics education and student programs to prepare citizen civic engagement. There is an imbalance between ideal conditions regarding the protection of citizens who should feel safe, especially from acts of sexual violence. However, in reality, this problem still occurs a lot, so a solution must be found, especially in tertiary institutions. This can be done by sharing efforts both in-class activities through various campus programs and in-classroom learning.

Research Methods

Research on anti-sexual violence education in tertiary institutions uses the literature study method. Literature study research methods are included in the qualitative approach. This research study originates from journal articles and books as well as documents regarding the urgency of anti-sexual violence education for students, which is very important as a provision in people's lives. It also contains forms of sexual violence education that can be carried out in tertiary institutions.

The results of the study were analyzed using descriptive analysis, namely describing the facts from the research results regarding the urgency of anti-sexual violence education, and continued with literature analysis, namely books and journal articles that were relevant, as well as various documents to produce an in-depth study.(Creswell, 2014).

Results and Discussion

The Urgency of Anti-Sexual Violence Education in Higher Education

The results of the study show that anti-sexual violence education in tertiary institutions is veryimportant because there are many cases of sexual violence in Indonesia which involve various components in tertiary institutions. The position of higher education is very vital to prepare the next generation who are ready to enter society. Various factors of sexual violence can occur. This can be seen from various theories of justice and human rights, as well as gender equality. Sexual violence is strongly influenced by various factors,including gender equality. There are various theories about gender equality, namely the theories of nurture, equilibrium, and nature. Gender equality has three theories, namely nurture, equilibrium, and nature. This nurture theory describes the equality of tasks and roles of women and men. This is relatedto the equality of quantity and trying to fight for it. Related to the social and cultural construction that causes differences between women and men causes many women to lag behind men not only in the family sphere but also within the community and also the state. Then the next theory is the theory of equilibrium which describes a harmonious concept of men and women. Women and men must work together, especially in life in the family or society as well as in the nation and state. (Zham-Zhamet et al., 2022).

Conventionally, a person's sexual status is determined by the human organs. This is considered a criterion of gender. This is related to gender. Gender is not sex which is a general term that refers to masculinity or femininity, including genital sex. Gender identity will determine gender roles like two sides of a coin (Money, 2008). Gender is closely related to social roles. This key concept forms the basis of the role of socialtheory. The social theory also describes the social roles of both men and women that produce gender stereotypes and influence gender behaviour. This can lead to the dominance of one gender (Schneider & Bos, 2019). This shows that there are factors that trigger violence by one gender because of stereotypes of domination that give rise to the seeds of sexual violence. For this reason, it is very important to raise awareness of gender equality so that there are no more feelings of superiority and domination.

Sexual violence is a form of violation of human rights. Human rights should be upheld bythe state, especially in a democratic country. This human right is also included in sexual rights. The existence of protection that guarantees justice and anti-sexual violence is something that must be Carried out by the state and grown for all its citizens, including in the education sector. The sexual minority group or various forms of discrimination and violence must be eradicated in Indonesia. (Novarin et al., 2020).

Forms of Anti-Sexual Violence Education Efforts in Higher Education

Forms of anti-sexual violence education efforts can be carried out by developing anti-sexual violence learning models in tertiary institutions and through various programs that encourage the activeinvolvement of students in efforts to support anti-sexual violence. This can be done by all tertiary academics. Forms of learning innovation in tertiary institutions that incorporate anti-sexual violence valuescan be carried out. Because through learning in class, students will gain knowledge and values regarding anti-sexual violence. This can be done by lecturers and various subjects in tertiary institutions.

The form of a learning model that can be used to foster anti-sexual violence can also be done with a service-learning model that allows active participation of students in society with various cases of sexual violence. Service learning is a concept in educational studies. The concept of service learningis closely related to learning activities by considering the needs of the community. The activities carriedout are the result of the actualization of learning in class. Students are active in activities that refer to service in the community. Activities that are based on volunteerism by paying attention to the benefits of others. To get to the voluntary stage, you have to look at the condition of the community and observethe situation in the community. Students who wish to find out what is happening in society and make students want to change environmental conditions to make it better. The application of knowledge at apractical level is the spearhead of service learning. Service learning is interpreted as education that prioritizes the experiences of students with various activities carried out to be able to address human needs in society together with a structured opportunity designed for the development of student learning. (Jacoby, 2009).

Citizenship attitudes can be formed with the service learning method. Not only attitudes, but the service learning method can also form beliefs, knowledge and skills that can develop students' abilities to become agents of change in society. The element of knowledge is very important and playsan important role in civic engagement. With knowledge, students can determine solutions to every problem in the environment. (Rimm-Kaufman et al., 2021).

Project-based learning (PBL) is a typical learner-centred learning model with student autonomy. Constructive analysis, goal setting, collaboration, communication, and reflection are packaged in real conditions on the ground. The PBL model has been explored in various contexts and at various stages of schooling. This method has been adopted from elementary education to higher education. Most of the studies reviewed were based on a pretest-posttest and quasi-experimental design with some baseline equivalence set but no random allocation of participants to the control and experimental groups, and the results, in the form of a causal relationship between PBL instruction andpositive student outcomes, were not can be determined with certainty. Things that help student learningin the form of modern digital technology, group processes, effective abilities in providing guidance and support, a balance between instruction with in-depth inquiry methods and harmonized assessments have been identified as influencing factors in the implementation of PBL. (Kokotsaki et al., 2016).

The consideration for choosing a learning model lies in the new knowledge that is created as a result of experiences that have been transformed. Creation of new knowledge, students show that a new experience will produce a new understanding also related to the contextual depth of the experience. This experience can be realized with digital technology that is utilized in the learning process (Gallagher, Renner, & Glover-Rijkse,

2020).

Programs that are included in various student organizations can also be carried out to foster the character of anti-sexual violence in tertiary institutions. This form of involvement can be a concrete manifestation of student participation in grounding anti-sexual violence within the campus environmentand in society. This participation is seen as student involvement which can be referred to as civic engagement. Civic Engagement is a way to lead to the formation of good citizens because good citizenswill rely on and be reflected in the behaviour of citizens who are part of human beings. Civic engagement is social capital to form good citizens. An ideal community and a stronger democracy with the involvement of its citizens (Theiss-Morse & Hibbing, 2005). Citizen involvement is a general.

The term refers to individual activities independently or as part of a community group, which has a focus on developing community knowledge and its political system, another form of involvement by identifying and seeking solutions to social problems in society. This involvement is carried out to gain benefits for the community and engage in constructive deliberations as members of the community about the political system and social problems in society, accompanied by finding solutions to community problems. (McClellan, 2013). However, Civic engagement is an indispensable value for a country. Civic engagement can be grown through civic education learning. Citizenship education is ableto improve various attitudes and actions of citizen involvement. This involvement must be included in the civic education learning curriculum. (Norell, 2022).

Conclusion

Anti-sexual violence education for students in tertiary institutions is very important and can be carried out in various ways to strengthen students' knowledge and involvement in dealing with variouscases of sexual violence. Efforts that can be made are to insert anti-sexual violence values into variousstudent programs. Another effort is to innovate various learning models, such as the service learning model, to encourage student involvement in solving various cases of sexual violence or being involvedin various efforts to prevent it.

Scientific Ethics Declaration

The authors declare that the scientific ethical and legal responsibility of this article published in EPHELS journal belongs to the authors.

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Reference

Arsyati, A. M., Pratomo, H., Ismail, I., Prasetyo, S., & Damayanti, R. (2017). Pengembangan media cetak pendidikan pencegahan kekerasan seksual balita di kota bogor. *Hearty, 5*(1). https://ejournal.uika-bogor.ac.id/index.php/Hearty/article/view/1052/866

Creswell, J. W. (2014). *Penelitian kualitatif & desain riset edisi* 3. Yogyakarta: Pustaka Pelajar.

Flanagan, C., & Levine, P. (2010). Civic engagement and the transition to adulthood. *Future of Children, 20*(1), 159–179.

Gallagher, V. J., Renner, M. M., & Glover-Rijkse, R. (2020). Public address as embodied experience: using digital technologies to enhance communicative and civic engagement in the communication classroom. *Communication Education, 69*(3), 281–299.

Hilmi, M. F., & Airlangga, U. (2019). Kekerasan seksual dalam hukum internasional. *Jurist- Diction, 2*(6), 2199– 2218.

Jacoby, B. (2009). *Civic engagement in higher education: Concepts and practices*. John Wiley & Sons.

Kokotsaki, D., Menzies, V., & Wiggins, A. (2016). Project-based learning: A review of the literature. *Improving Schools, 19*(3), 267–277.

McClellan, E. F. (2013). Review of teaching civic engagement: from student to active citizen. *Journal of Political Science Education, 9.*

Money, J. (1985). Gender: History, theory and usage of the term in sexology and its relationship to nature/nurture. *Journal of Sex & Marital Therapy*, *11*(2), 71-79.

Norell, E. (2022). Civic engagement meets service learning: Improving Wikipedia's coverage of state government officials. *PS - Political Science and Politics, 55*(2), 445–449.

Novarin, A. S., & Pattipeilhy, S. C. H. (2020). Feminism perspective in understanding human rights issues of Queer Group in Semarang City, Indonesia. *Jurnal HAM*, *11*, 487.

Novarin, A. S., & Pattipeilhy, S. C. H. (2020). Feminism perspective in understanding human rights issues of queer group in semarang city, Indonesia. *Jurnal HAM*, *11*, 487-504.

Novarin, A. S., & Pattipeilhy, S. C. H. (2020). Perspektif feminisme dalam memahami permasalahan hak asasi manusia kelompok Queer di Kota Semarang, Indonesia. *Jurnal Ham*, *11*(3), 487-504.

Rahman, N., & Sarip, S. (2021). Kebijakan perlindungan anak korban kejahatan seksual di cirebon. *Jurnal Hukum & Pembangunan*, *50*(3), 619-632.

Rimm-Kaufman, S. E., Merritt, E. G., Lapan, C., DeCoster, J., Hunt, A., & Bowers, N. (2021). Can service-learning boost science achievement, civic engagement, and social skills? A randomized controlled trial of Connect Science. *Journal of Applied Developmental Psychology*, *74*, 101236.

Schneider, M. C., & Bos, A. L. (2019). The application of social role theory to the study of gender in politics. *Political Psychology*, *40*, 173-213.

Theiss-Morse, E., & Hibbing, J. R. (2005). Citizenship and civic engagement. *Annu. Rev. Polit. Science*, *8*, 227-249.

Zeng, J. (2020). Metoo as connective action. Zurich open repository and archive university of zurich main library, *14*, 171–190.

Zham-Zham, L. M., Sugiri, B., & Sulistyarini, R. (2022). Telaah kritis pengaturan pornografi di indonesia dalam perspektif teori kesetaraan gender. *Jurnal Ilmiah Pendidikan Pancasila dan Kewarganegaraan*, *7*(1), 49-56.

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Nutritional Status, Iron Status (Hb) and Attitude to Reproductive Health as Components of Adolescent Reproductive Readiness

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Abstract: The fertility rate for adolescents aged 15-19 years in Indonesia reaches 10%. Approximately 1.4 million (30%) young women aged 15-19 years have sexual intercourse, which results in pregnancy and around 57% of these pregnancies are unwanted. The tendency to get married and get pregnant at a young age, whether planned or not, results in reproductive readiness, both mental and physical, which is very important for young women. Their nutritional status measures adolescents' physical readiness—meanwhile, reproductive knowledge and perceptions of reproductive health measure adolescents' mental readiness for reproduction. The study aimed to identify risk factors for nutritional status, iron status and reproductive health attitudes. This type of research is analytic observational with a cross-sectional design. The population of this study was all adolescents in Ogan Komering Ilir District. The sampling technique used was cluster sampling. The research sample was 139 young girls at SMA N 1 Kayuagung, Ogan Komering Ilir Regency, South Sumatra. Data is presented in univariate and bivariate. The statistical test was chi-square with the Fisher's exact/Kolmogorov Smirnov alternative test. The results showed that around 52.5% of female adolescents were underweight, 15.6% had anaemia, and 6.5% had poor reproductive health attitudes. Based on bivariate analysis, risk factors related to adolescent iron status were eating habits (p-value = 0.039), risk factors related to adolescent attitudes were class level (p-value = 0.0005), father's education level (p-value = 0.017), puberty age (p-value = 0.008) and level of knowledge (p-value = 0.003). Education is needed to increase reproductive health knowledge and improve adolescent eating habits intensively with exciting and not dull material such as role-playing or group discussions.

Keywords: Reproductive readiness, Adolescents, Nutritional status, Haemoglobin, attitude

Introduction

Adolescence is a period of transition from childhood to adulthood (Kementerian Kesehatan RI, 2015) The process of rapid growth and reproductive development in adolescents causes adolescents to have a great curiosity and dare to take risks without careful consideration (Soetjiningsih et. al. 2004) The magnitude of the risk of adolescent behavior is not in line with information related to pregnancy unwanted and sexually transmitted diseases received by adolescents (Susanto & Rahmawati, 2016).

The fertility rate for adolescents aged 15-19 in Indonesia reaches 10% (Departemen Kesehatan RI, 2004). Approximately 1.4 million (30%) female adolescents aged 15-19 have sexual intercourse which results in pregnancy and approximately 57% of these pregnancies are unwanted (Soetjiningsih et. Al, 2004). IDHS report

in 2012, the fertility rate for the 15-19 year age group was 48 births per 1000 women. Pregnancy at a young age can be at risk of premature birth, low birth weight (LBW), and childbirth bleeding, which can increase maternal and infant mortality. The 2012 IDHS found that neonatal, postneonatal, infant and under-five mortality rates for mothers aged less than 20 years were higher than those for mothers aged 20-39 years (Kementerian Kesehatan RI, 2015).

The consumption patterns of adolescents who tend to limit themselves and are low in nutrients have an impact on their nutritional status (Rahmiwati, 2013). A study of 1,200 young women in Bengkulu City found that 79.2% of adolescents' diets were not good and the prevalence of anemia was 43% (Suryani et al, 2017). The prevalence of anemia was > 40%. including severe category problems (WHO, 2007). Unhealthy eating patterns result in adolescents who are very thin due to malnutrition (malnutrition) and in the long-term Chronic Energy Deficiency (CED). SEZ is a condition where there is a chronic lack of energy and protein nutritional intake which is characterized by an upper arm circumference of LLA <23.5 cm (Departemen Kesehatan RI, 2002). KEK women are at risk of giving birth to children who are LBW or low birth weight < 2500 grams. Prevalence of risk of KEK in women of childbearing age is generally 20.8% nationally and the prevalence of CED risk in the 15-19 group who were not pregnant increased from 30.9% in 2007 to 46.6% in 2013 (Kementrian Kesehatan Republik Indonesia. 2013).

The tendency to get married or get pregnant at a young age, whether planned or not, results in reproductive readiness, both mental and physical, which is very important for young women. Adolescents' physical readiness is measured by their nutritional status. Meanwhile, adolescents' mental readiness for reproduction is measured by reproductive knowledge and adolescent perceptions of reproductive health (Matodipuro, 2000). In 2012, there were 57 cases of infant mortality in Ogan Komering Ilir (OKI) District, 48 cases were neonatal deaths and LBW caused 31% of these neonatal deaths. In the same year 2012, in Ogan Komering Ilir (OKI) District, 17 maternal deaths were recorded, with 35% due to bleeding. In 2012, the number of pregnant women with KEK in OKI Regency was 561 people or 2.94% of the number of pregnant women.

The magnitude of the risk of teenage pregnancies needs to be anticipated with the reproductive readiness of young women in terms of nutritional status, reproductive knowledge, perceptions of reproduction, and others. However, the typical characteristics of adolescents and the tendency for poor consumption patterns push adolescents into health problems such as CED and anemia. So that it can contribute to the problem of LBW and bleeding in maternal and child mortality. Seeing the magnitude of the nutritional problems in pregnant women, LBW problems and bleeding that occur in OKI District which results in maternal and child deaths, it is necessary to identify the contribution to the reproductive readiness of young women in OKI District in these problems.

Results and Discussion

Reproductive readiness was examined through variables of nutritional status, iron status (Hb) and adolescents' attitudes towards reproductive health. Based on Table 1, around 52.5 % of young women are underweight, 39.6% are normal weight and 7.9% are categorized as obese. Based on iron status or blood hemoglobin levels, 16.5% had anemia. Only 6.5% of adolescents have poor reproductive health attitudes. Based on Table 1, it can also be seen that a majority of teenagers are at the level/class 2 of SMA, as much as 46.0%. The teenagers, on average, came from fathers and mothers with tertiary education as much as 79.9% and 83.5%. Also, the average comes from fathers and mothers who work as employees as much as 96.4% and 50.4%. The majority of adolescents experience puberty at the age of ≥12 years, as much as 82.7%. Adolescents who do not receive information about reproductive health are 46.8%. In addition, most of these teenagers have bad eating habits, as much as 66.2%. Most of these teenagers live with their families, as much as 94.2%. Also, the average knowledge of adolescents about reproductive health is still lacking at 78.4%.

Table 2 shows the relationship between risk factors and the nutritional status of adolescents. From the results of the bivariate analysis, it can be seen that there are no risk factors related to the nutritional status of adolescents at SMA Negeri 1 Kayuagung. Table 2 also shows that for each of the characteristics of the respondents, the group with abnormal nutritional status (thin or fat) was still dominated, such as poor eating habits, 63% of whom came from the abnormal body group. Based on Table 3, it can be seen that the risk factor associated with adolescent iron status is eating habits (p-value = 0.039). The proportion of adolescents with poor eating habits and experience anemia is 21.7%, while adolescents with good eating habits and who experience anemia is only 6.4%. Adolescents who have poor eating habits are at risk 3,406 times higher for anemia compared to adolescents who have good eating habits.

Tabel 1. Distribution of respondent characteristics

Variabel	Total (n)	Percent (%)
Nutritional Status		
Thin	73	52,5
Normal	55	39,6
Fat	11	7,9
Iron Status		
Anemia	23	16,5
No Anemia	116	83,5
Reproductive Health Attitudes		
Poor	9	6,5
Good	130	93,5
School level		
Level 1	39	28,1
Level 2	64	46,0
Level 3	36	25,9
Father's Education		
Low	28	20,1
High	111	79,9
Mother's Education		
Low	23	16,5
High	116	83,5
Father's Job		
No working/labor	5	3,6
Employee	134	96,4
Mother's Job		
No working/labor	69	49,6
Employee	70	50,4
Puberty Age		
≥12 years	115	82,7
< 12 years	24	17,3
Source of Information		
No Adekwat	65	46,8
Adekwat	74	53,2
Eating habit		
Poor	92	66,2
Good	47	33,8
Residence		
Not With Family	8	5,8
With family	131	94,2
Knowledge		
Poor	109	78,4
Good	30	21,6

Table 2. Results of bivariate analysis of the relationship between risk factors and nutritional status

Variables	Nutrition Status				*p-value*	PR(95% CI)
	Poor		Good			
	n	%	n	%		
School level						
Level 1	24	51,5	15	38,5	0,886	0,886 (0,637-1,232)
Level 2	35	54,7	29	45,3	0,788	0,788 (0,577-1,075)
Level 3	25	69,4	11	14,2	-	Ref
Father's Education						
Low	16	57,1	12	42,9	0,856	0,933 (0,655-1,328)
High	68	61,3	43	38,7		
Mother's Education						
Low	13	56,5	10	43,5	0,852	0,923 (0,627-1,359)
High	71	61,2	45	38,8		
Father's Job						
No working/labor	3	60,0	2	40,0	1,000	0,993 (0,479-2,057)

Employee	81	60,4	53	39,6		
Mother's Job						
No working/labors	44	63,8	25	36,2	0,532	1,116 (0,852-1,462)
Employee	40	57,1	30	42,9		
Source of Information						
No Adekwat	35	53,8	30	46,2	0,189	0,813 (0,616-1,074)
Adekwat	49	66,2	25	33,8		
Eating habit						
Poor	58	63,0	34	37,0	0,485	1,140 (0,844-1,540)
Good	26	55,3	21	44,7		
Residence						
Not With Family	4	50,0	4	50,0	0,712	0,819 (0,404-1,659)
With family	80	61,1	51	38,9		

* Sig. <0,05

Table 3. Results of bivariate analysis of the relationship between risk factors and iron status

Variabel	Status Besi					
	Anemia		Tidak Anemia		*p-value*	PR(95% CI)
	n	%	N	%		
School level						
Level 1	4	10,3	35	89,7	0,273	0,462 (0,152-1,403)
Level 2	11	17,2	53	82,8	0,726	0,773 (0,343-1,746)
Level 3	3	22,2	28	80,0		Ref
Father's Education						
Low	2	7,1	26	92,9	0,164	0,378 (0,094-1,516)
High	21	18,9	90	81,1		
Mother's Education						
Low	4	17,4	19	82,6	1,000	0,062 (0,398-2,832)
High	19	16,4	97	83,6		
Father's Job						
No working/labors	0	0	5	100	0,590	
Employee	23	17,2	111	82,8		
Mother's Job						
No working/labors	12	17,4	57	82,6	0,970	0,107 (0,524-2,337)
Employee	11	15,7	59	84,3		
Puberty Age						
≥12 years	21	18,3	94	81,7	0,366	0,191 (0,550-8,729)
< 12 years	2	8,3	22	91,7		
Source of Information						
No Adekwat	8	12,3	57	87,7	0,302	0,607 (0,275-1,339)
Adekwat	15	20,3	59	79,7		
Eating habit						
Poor	20	21,7	72	78,3	0,039	0,406 (1,066-10-,881)*
Good	3	5,4	44	93,6		
Residence						
Not With Family	1	12,5	7	87,5	1,000	0,744 (0,114-4,842)
With family	22	16,8	109	83,2		

* Sig. <0,05

Based on the results of the multivariate analysis in table 5, it can be seen that eating habits have a significant relationship with iron status (p-value <0.05). Adolescents with poor eating habits have a 0.247 times lower risk of experiencing anemia compared to adolescents who have good eating habits after controlling for sources of information, school level, father's education, mother's education, and mother's occupation. With a 95% degree of confidence, in the general population, researchers believe that poor eating habits are a protective factor against iron status with a range of 0.066 to 0.933 (PRAdjusted = 0.247; 95% CI = 0.066-0.933).

Based on Table 4, it can be seen that the risk factors associated with adolescent attitudes are school level (p-value = 0.005), father's education (p-value = 0.017), puberty age (p-value = 0.008), and knowledge (p-value = 0.003). The proportion of adolescents with a good attitude tends to increase in the group at a higher level/class. Adolescents in grade 1 have a 0.795 times lower risk of unfavorable attitudes than grade 3 adolescents. The

proportion of adolescents with low fathers' education and poor attitudes is 17.9%—meanwhile, only 3.6% of adolescents with a high father's education and good attitude. Adolescents with low paternal education are at risk 4.955 times more for having unfavorable attitudes compared to adolescents with high paternal education

Table 4. Results of bivariate analysis of the relationship between risk factors and adolescent attitudes

Variables	Attitude				*p-value*	PR(95% CI)
	Poor		Good			
	n	%	n	%		
School level						
Level 1	8	20,5	31	79,5	0,005	0,795 (0,678-0,932)*
Level 2	1	1,6	63	98,4	1,000	0,984 (0,954-1,015)
Level 3	0	0	36	100	-	Ref
Father's Education						
Low	5	17,9	23	82,1	0,017	4,955 (1,423-17,257)*
High	4	3,6	107	96.4		
Mother's Education						
Low	1	4,3	22	95,7	1,000	0,630 (0,083-4,801)
High	8	6,9	108	93,1		
Father's Job						
No working/labor	0	0	5	100	1,000	-
Employee	9	6,7	125	93,3		
Mother's Job						
No working/labor	4	5,8	65	94,2	1,000	0,812 (0,227-2,896)
Employee	5	7,1	65	92,9		
Puberty Age						
≥12 years	4	3,5	111	96,5	0,008	0,167 (0,048-0,576)*
< 12 years	5	20,8	19	22,4		
Source of Information						
No Adekwat	5	7,7	60	92,3	0,734	1,423 (0,399-5,077)
Adekwat	4	5,4	70	94,6		
Eating habit						
Poor	0	0	8	100	1,000	-
Good	9	6,9	122	93,1		
Knowledge						
Poor	3	2,8	106	97,2	0,003	0,138 (0,037-0,518)*
Good	6	20,0	24	80,0		

* Sig. <0,05

The proportion of adolescents who experience puberty at the age of ≥ 12 years and have a poor attitude is 3.5%. Meanwhile, adolescents who experience puberty at the age of <12 years and have less attitude are 20.8%. Adolescents who experience puberty at the age of ≥ 12 years have a 0.167 times lower risk of having poor attitudes than adolescents who experience puberty at the age of <12 years. The proportion of adolescents with less knowledge and less attitudes is 2.8%. While adolescents with sufficient knowledge and have less attitude, as much as 20.0%. Adolescents with less knowledge have a 0.138 times lower risk of having poor attitudes than adolescents with good knowledge.

Readiness is an individual state that supports it so that it is ready to respond or respond in the best way to the conditions that are happening (Slameto, 2015). Reproductive readiness is the most important developmental task in adolescence. Reproductive readiness consists of physical readiness as measured by the nutritional status of adolescents and mental readiness as measured by reproductive knowledge and adolescent perceptions related to reproductive health. Nutritional status is the state of the body that is influenced by food consumption, absorption of nutrients, and utilization of food nutrients. anthropometric, biological, clinical, and ecological factors and food consumption can be carried out (Riyadi, 2001). Assessment of nutritional status can be carried out by anthropometric, biological, clinical, and ecological factors and food consumption (Gibson, 2005). The results showed that more than half of the respondents weighed 52.5% thin and 7.9% body weight categorized as fat. A similar study also reported that 49.6% of young women aged 12-14 years in the city of Surabaya were undernourished (BMI <18.5 kg/m2) (Santy, 2006). Waryana (2010) stated that a lack or excess of nutrition in the long term could hurt health. Adolescents who are very thin due to malnutrition often called malnutrition, if it lasts too long will result in Chronic Energy Deficiency (KEK) (Departemen Kesehatan RI, 2002). SEZ women are at risk of giving birth to children with LBW or Low Birth Weight < 2500 grams (Lubis, Z. 2003).

Nutritional status is influenced by various factors including energy and nutrient intake, gender, education, fiber consumption habits, physical activity and parents' genetic factors. BMI nutritional status. These results need to be investigated further by examining the variable intake/consumption of adolescents and also the activities of adolescents.

Poor eating habits are a common cause of nutritional problems in adolescents such as eating irregularly, avoiding certain foods, reducing meal frequency, and consuming unbalanced food (Zofiran et al, 2011The research results also show that the eating habits of adolescent girls are related to the incidence of anemia and around 15.6% of adolescent girls who were respondents experienced anemia. Adolescence is a period of growth and development, both physically, mentally and in activity, so the need for nutritious food is great (Zong, 2014), Nutritional anemia in adolescents contributes to increasing maternal mortality, LBW babies, and prenatal mortality. So to break the life cycle of malnutrition is to increase iron nutrition for young women (Kaur, 20016). Good eating habits can prevent anemia in young women, including consuming animal protein, eating fruit rich in vitamin C, consuming home-cooked food, and choosing nutritious snacks (Akib, 2017).

Conclusion

Approximately 52.5% of female adolescents are underweight, 15.6% are anemic and 6.5% have poor reproductive health attitudes. Poor eating habits are a protective factor against iron status/anemia (PR Adjusted = 0.247; 95% CI = 0.066-0.933). Age of Puberty and knowledge are the most dominant risk factors for reproductive health attitudes.

Recommendations

Education to increase reproductive health knowledge and improve adolescent eating habits intensively can increase the degree of reproductive readiness of adolescents. Further research is needed with more complete independent variables and on a larger sample.

Scientific Ethics Declaration

The authors declare that the scientific ethical and legal responsibility of this article published in EPHELS journal belongs to the authors.

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References

Akib, A., & Sumarmi, S. (2017). Kebiasaan makan remaja putri yang berhubungan dengan anemia: kajian positive deviance food consumption habits of female adolescents related to anemia: A positive deviance approach. *Amerta Nutrition*, *1*(2), 105-116.

Depkes, R. I. (2002). *Pedoman penanggulangan Ibu Hamil Kekurangan energi kronis. Jakarta*: Direktorat Pembinaan Kesehatan Masyarakat.

Depkes, R.I. (2004). *Pedoman pelayanan kesehatan reproduksi remaja*. Jakarta: Direktorat Pembinaan Kesehatan Masyarakat.

Gibson, R.S. (2005). *Principles of nutritional assessment* (2 nd ed.). Oxford [GB]: University Press.

Kaur, S., Deshmukh, P. R., & Garg, B. S. (2006). Epidemiological correlates of nutritional anemia in adolescent girls of rural Wardha. *Indian Journal of Community Medicine*, *31*(4), 255.

Kementrian Kesehatan, R.I. (2013). *Riset kesehatan dasar*. Jakarta: Badan Penelitian dan Pengembangan Kesehatan Kementrian Kesehatan Republik Indonesia.

Kementerian kesehatan RI. (2015). *INFODATIN : Situasi kesehatan reproduksi remaja*. Jakarta: Pusat Data dan Informasi Kemeterian Kesehatan RI

Lubis, Z. (2003). *Status gizi ibu hamil serta pengaruhnya terhadap bayi yang dilahirkan*. Program Pascasarjana Institut Pertanian Bogor.

Manuaba, I. A. C. (2009). *Memahami Kesehatan reproduksi wanita*(2nd ed.). Jakarta: Egc.

Matodipuro, S. (2000). Pengetahuan dan Pandangan Remaja Wanita tentang Kesehatan Reproduksi. *Jurnal Kedokteran dan Farmasi Medika 3*(26), 158-165.

Rahmiwati, A. (2013). Kesiapan reproduksi remaja putri di sekolah menengah atas dan pondok pesantren: Manakah yang Lebih Baik?. *Jurnal Ilmu Kesehatan Masyarakat*, *4*(3).

Riyadi, H. (2001). *Metode penilaian status gizi secara antropometri* [diktat]. Bogor(ID): Institut Pertanian Bogor.

Santy, R. (2006). Determinan indeks massa tubuh remaja putri di kota bukit Tinggi, Tahun 2006. *Kesmas: Jurnal Kesehatan Masyarakat Nasional (National Public Health Journal)*, *1*(3), 134-138.

Slameto, P. (2015). *Belajar dan faktor-faktor yang mempengarhi*. Jakarta: Rineka Cipta.

Soetjiningsih, S. (2004). *Tumbuh kembang remaja dan permasalahannya*. Sagung Seto. Jakarta, 320.

Stevens, G. A., Finucane, M. M., De-Regil, L. M., Paciorek, C. J., Flaxman, S. R., & Branca, F. Assessing the iron status of populations. Report of a joint World Health Organization/centers. *Trends Cell Biol 201424*(5), 30312.

Suryani, D., Hafiani, R., & Junita, R. (2017). Analisis pola makan dan anemia gizi besi pada remaja putri Kota Bengkulu. *Jurnal Kesehatan Masyarakat Andalas*, *10*(1), 11-18.

Susanto, T., & Rahmawati, I. (2016). A community-based friendly health clinic: An initiative adolescent reproductive health project in the rural and urban areas of Indonesia. *International Journal of Nursing Sciences*, *3*(4), 371-378.

Waryana. (2010). *Gizi reproduksi.* Yogyakarta (ID): Pustaka Rihama.

Woodruff, B. A., & Duffield, A. (2000). *Assessment of nutritional status in emergency-affected populations.* Geneva: United Nations/Sub-Committee on Nutrition.

Zofiran, N. S., Kartini, M., Siti Sabariah, B., & Ajau, D. (2011). The relationship between eating behaviours, body image and BMI status among adolescence age 13 to 17 years in Meru, Klang, Malaysia. *American Journal of Food Nutrition, 1*(4), 185-92.

Zong, X. N., & Li, H. (2014). Physical growth of children and adolescents in China over the past 35 years. *Bulletin of the World Health Organization, 92*, 555-564.

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Analysis of Sexual Health Education in the Film "Dua Garis Biru" and its Urgency on Moral Internalization for Adolescents

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Abstract: Teenagers now experience a flood of information that continues to overflow every day. In the hands of teenagers, sex information is now easy to get. Even for those who don't intend to look for it, sometimes sex information circulates on their search engine homepage. It is so easy to find sex information, exposing teenagers to negative perceptions about sex. This is because most of the circulating sex information leads to pornography. This research raises one of the themed films of sex education, entitled "Dua Garis Biru" which was made to ward off misinterpretation of sex. This is considered by some as a bright spot to make people aware of the importance of sex education to build morale for their children. This study aims to identify sex education in the film "Dua Garis Biru". In addition, the depicted adolescent personalities were analyzed using the basis of their psychosocial and psychosexual theories. After that, identify how the process of moral internalization in adolescents by basing it on their cognitive development model. All of these theories will explain how sex is related to culture, and then affect moral development. This research is qualitative research using observation and documentation techniques in data collection. It used content, semiotic, and framing analysis which used sexual health, and social approaches. The results obtained from this study show that there are values of sex education related to moral internalization in the film "Dua Garis Biru". The value of sex education is contained in several messages such as the meaning of virginity, the danger of abortion, the reality of pregnancy in adolescents, and the negative effects of early marriage.

Keywords: Sexual health, Education, Film, Moral internalization, Adolescent.

Introduction

Adolescents are always growing in amazing ways. Even their characteristics are always different from when their parents grew up. The influence of the development of technology, industry, globalization, and the era of disruption makes the development of millennial children more quickly mature (O'Hara, 2010, p. 110). But not a few adolescents who experienced irregularities, one deviation that occurs is a misperception about how to foster

relationships between men and women (Tyson, 2011, p. 40). At present, in our society, there is a “bucin” phenomenon (a.k.a. Slave of Love). It’s not taboo when they are having sex at a young age. This is tough to do due to the negative impact of the ease of accessing unlimited information. Nearly 60% of children are good at using gadgets, and some of them are addicted to them. And it’s caused emotional and behavioral disorders (Pfundmair et al., 2015, pp. 228–233). If not really taken seriously, this will become a silent killer for the nation’s future. Slaves love or "bucin" is a term for those who are in a drunken romance, and seem willing to make any sacrifice. Of course, this is often misinterpreted by teenagers as something that gives each other pleasure without committing and knowing what they will bear after having a child (Bowmaker & Emerson, 2013, p. 3155).

In essence, this matter is about responsibility and mutual respect. In almost all schools there are cases of pregnancy before marriage, due to free sex (Kamal & Ulas, 2022, p. 1189). Many cases end in early marriage or abortion of the fetus, and cervical cancer (Vaisy, 2014, p. 270). Apart from the graphic data statistics related to this case, the existence of this case should be enough reason for us to fight for sexual health education. This is an effort to liberate young people against a bleak future. Many parents do not know about the importance of sexual health education itself, so they submit this task to the school (Cameron et al., 2020, p. 540). In some schools, sexual health education efforts have been carried out through counseling and modeling from educators (Khoei et al., 2022, p. 566). But this is still less effective if seen in reality by many of the teens who are still dating secretly or also referred to as dating on the back street. Some of the teenagers after being interviewed claimed that this was able to meet their needs to get love and attention. Of the 13 teenagers in the study group, there were 11 teenagers who claimed to be dating. Whereas 2 other teenagers claimed that they felt disgusted by dating, while others claimed they were strictly forbidden by their parents (Indrayani & Jalil, 2016, p. 15). This is where the evidence is revealed that the actual role of sex education should be greater in parents.

This problem becomes more complex because many of us are confused about how to teach sexual health education. This is because adolescents are still developing subjects with all their biological and psychological aspects. Naturally, in adolescents, there is a change in body shape due to puberty and the turmoil of attraction towards the opposite sex. There must be special treatment for adolescents tailored to each unique condition (Formanek & Gurian, 1980, p. 28). The material provided must also be of a medium so as not to encourage overflowing turmoil, and not too minimal information to adequately satisfy their curiosity. Many of us are still struggling about sexual health education because we are confused about how to teach it. Whereas in some books such as the *Qurratul ‘uyun, Uqutudh Dillijain, Risalatul Mahid*, etc., have taught the importance of sex education itself (Nurish, 2010, p. 270). This indicates that previous scholars highly upholds sex education as a learning. But over the times, such literacy is largely abandoned and rarely preserved. Many obstacles are at the root of the problem including a decrease in the level of interest in reading, a reduction in people who are proficient in Arabic, and a monotonous way of teaching. Re-claiming this actually happened because of the era of disruption, where there were many fundamental changes to the social fabric. For example, learning is no longer convergent centered on educators, but divergent which spreads to all students. Speed of access to information also makes us reluctant to bother studying it now (Tangney, 2014, p. 272). Many of us who prefer the visualization of information in the form of color images rather than just a row of monochrome sentences (Lacković & Olteanu, 2020, p. 605). It must be admitted if this is the characteristic of almost all humans today. This is what we must mark as a step towards a renewal that must be done.

The main obstacle to sex education is the lack of educational literacy that addresses it. Very little about books, journals, soap operas, blogs, and even advertisements that make sex education as the main topic (Attwood et al., 2015, p. 530). Some heroic actions from some young people of the nation are the ones who spearheaded the sex education conscious movement through educative videos on YouTube, blog posts, and even feature films. However, this is still inferior when compared to the massive movements of several soap operas and film that display a variety of less educative content. This can be seen from several scenes of courtship, jealousy, and even fighting to attract the audience's interest (Thompson & Yeboah, 2013, p. 377). From all the explanations above it can be concluded the end of the problem lies in the lack of learning resources and what methods are suitable for sex education for adolescents. This will be answered by the author to examine a story "Dua Garis Biru". This story was born due to answer the anxiety about freedom of sex that often occurs in adolescents. Two teenagers, Dara and Bima, were involved in an early marital relationship due to pregnancy out of wedlock. Here illustrates how the dilemma of these two teenagers made a decision that made his life unusual and show how important the role of the family. Uniquely, besides inviting a lot of praise, this film also invites cons from several groups. Many of them denounce this as a moral setback. It is very contradictory to the goals of Gita, the creator of the story. He intended this film to be present as an education on sex for teenagers. This indicates there are some miss interpretations of their meaning so a deeper analysis is needed related to moral internalization. Based on the background of the research above, then the problem can be formulated as following: What are the values of

sexual health education in the story of "Dua Garis Biru" and its urgency on moral internalization for adolescents?

Method

Type of Research

This type of research used is qualitative research, namely research that uses a naturalistic approach to find and understand a phenomenon in a specific context (Creswell, 2014, p. 13). During the design phase, it is imperative for researchers to possess a comprehensive understanding of the intended utilisation and analytical methodology for data collecting. This entails the consideration of many views, particularly when incorporating self-generated video data as a component of this methodology. Video can be utilised as a valuable tool in the development of novel protocols and research collaborations, extending beyond the mere replication of conventional instrumental methods. During the analysis phase, video footage can be utilised for a multitude of applications. As an illustration, a scholar may employ a continuous comparative technique to categorise emergent themes within video data, or they may opt to exclusively analyse the verbal content of a video dataset through the utilisation of Qualitative Content Analysis. One notable advantage of video research is in its inherent flexibility and multifaceted selection, which affords researchers a wider range of adaptable choices for subsequent dissemination. During the period of dissemination, researchers need to carefully consider the consumption of their video work, both inside and beyond the academic community, as it serves the purpose of research and frequently embodies creative elements. This encompasses the examination of both digital publishing and dissemination methods, alongside traditional norms and media employed for the dissemination of academic research. This book also emphasises the significance of effectively identifying and engaging with the appropriate target audience, particularly those who possess an interest in research findings. Furthermore, it underscores the necessity of broadening the audience reach from local to global, and from specific to generic demographics (Haris, 2016, pp. 121–129).

Analysis Techniques

This research uses 3 analysis techniques, namely:

a. Framing Analysis, this method was developed based on social construction theory. According to this theory, humans and society are seen as continuous dialectical, dynamic, and plural products. In this view, humans as individual beings have an influence on society which is a social organization. Vice versa, society gives social identity to humans as its members. Peter L. Berger calls it a moment that is divided into 3 events. First, externalization, namely the process of outpouring or expressing the human self into the world, both in mental and physical activities. Second, objectivation, namely the results that have been achieved both mentally and physically in previous externalization activities. These results are objective realities that can be experienced by everyone. Third, internalization is the re-absorption of the objective world into consciousness in such a way that individual subjective is influenced by the structure of the social world. According to Berger, reality is formed and constructed so that it has a plural meaning. This means that the truth revealed in this study will produce various meanings on the same problem by taking into account the cultural and individual backgrounds of each (Eriyanto, 2001, pp. 13–16).

b. Content Analysis, Material analysis is a common Communication Science method for assessing media content. Digitised media environment data have raised the importance and prevalence of this phenomena. Scholarly studies of news sources and issues, particularly agenda building and framing, have used automated content analysis (ACA). The content analysis process includes data retrieval, structuring and storage, cleansing, and analysis. Open data storage allows scalability, independence, and adaption. Example: JSON-based format. The initial step in data cleansing is replacing different expressions of key concepts with a common code. Using scenario-specific actor and concept lists can do this. Part-of-speech tagging (POS) and named entity recognition are also available at this level. Data analysis often uses word frequency-based methods such Log-Likelihood scores, PCA, co-occurrence or semantic networks, and cluster analyses. Academic research uses theme models including Latent Dirichlet Allocation (LDA), author-theme models, and structured topic models. Popular Python modules like NLTK, scikit-learn, and gensim can be used for analysis. This method makes the workflow visible and replicable. Modern communication science relies heavily on textual analysis. However, there is no reason to avoid using these methods to analyse visual material or other data. Content analysis framework creation goes

beyond technical tasks that can be outsourced. Proficiency requires technical skill and a thorough comprehension of the subject. (Trilling & Jonkman, 2018, pp. 2–14).

c. Semiotic Analysis, Semiotic analysis is an interpretive approach that entails the identification and examination of signs and symbols present in a given text or artistic creation, with the aim of comprehending its underlying meaning and the cognitive processes employed in its production. This methodology is based on the research conducted by Charles Peirce, who introduced a classification system consisting of three distinct categories of signs: icon, index, and symbol. Each of these types of signs exhibits a distinct association with the object it signifies. An icon is characterised by its resemblance to the object it represents, an index is distinguished by the presence of evidence indicating its object, and a symbol is a sign that is universally recognised and agreed upon to represent its object by convention. Within the realm of education, the application of semiotic analysis serves as a valuable tool for comprehending the cognitive processes of pupils, particularly in terms of their reasoning abilities and the construction of meaning. In a research investigation pertaining to students' comprehension of astronomy, a semiotic analysis was employed to scrutinise the utilisation of physiological and material processes by students in representing astronomical occurrences. The present investigation elucidated the manner in which students employed diagramming as a means to uncover the underlying structural connections among these events. Furthermore, it examined how their comprehension was influenced by the dynamic interaction between icons, indices, and symbols within their visual representations. The scope of semiotic analysis extends beyond visual forms and encompasses other types of representation, provided they adhere to Peirce's pragmatic and semiotic definition. The focal point of this approach lies in the dynamic process of "diagramming" as opposed to static "diagrams," hence emphasising the active involvement of the individual constructing meaning in the manipulation and observation of the diagram to develop novel insights (Ferguson et al., 2023, pp. 2–14).

Sources of Research

Sources of research data are everything in the form of people, places or goods or papers that can provide information or data needed in research. In this study (Setiawan, 2018, p. 15), researchers used two sources of data types:

a. Primary Data Sources, namely sources of materials or documents presented by the person or party concerned. Or data directly related to the object of research. The primary data source for this research is the film “Dua Garis Biru” with a duration of 1:52:17 seconds. The data were collected using the *mise-en-ścene*. Literally translated it means “to put on stage”. A useful definition might be the contents of the frame and the way that they are organized. *Mise-en-ścene* encompasses both what audiences can see, and the way in which we are invited to see it. The setting can be in the form of a place and atmosphere that gives the story behind the story so that it has a big influence on understanding social construction. Scene is a scene that is taken to prove an interaction or emotion between characters. In addition, the scene is also taken to determine the behavior figure so as to know the motivation underlying the action. Dialogue is a conversation between characters that liven up the atmosphere. Through dialogue we can find out what the character is struggling with (Gibs, 2012, p. 5).

b. Secondary Data Source, are data sources that complement primary data sources. Secondary data sources in this study are books, journals, previous researches, and other videos relevant to the research theme.

Literature Review

The Understanding of Sexual Health Education for Adolescent

Regarding the definition of sexual health education is the rest has been explained in the previous chapter. So, in this chapter we will only reaffirm and complete the definitions that are still lacking. Of course, as said David Campos to identify sex education globally is very difficult. This is because sex education is inseparable from the culture and culture of the community, even religion. Many experts explain the meaning of sex education based on the background. However, this research will certainly use theories that fit the author's background and environment. Sexual health education is still often overlooked because many of our society consider it taboo. Plus, the role of education itself is still underdeveloped while the problems around it are increasingly worrisome. This can be seen from the content of sex education which is still in the form of a biological reproductive system as well as about social normative norms. Besides the massive discussion about sex education there are still many who are directed at children, because teenagers can still be categorized as the like.

Though between the two have different characteristics and needs. Therefore, it is important for us to understand what the concept of sex education is right for adolescents.

Sexual health education is a clause consisting of two words elements, namely "education" and "sex". If it is logical, of course we have understood the meaning of the word education as a learning process from not knowing to knowing. But it's not quite right if it hasn't heard what Ki Hadjar Dewantara said about education. According to him education is an effort to advance the growth of character (inner strength, character), mind (intellect), and the body of a child, in order to live in harmony with his world. So, what are the criteria for character, mind, and body of the child? This is how Ki Hadjar Dewantara gave his explanation,

First, about Indonesian people who are virtuous are those who have inner strength and character. That is, education is directed to improve the image of humans in Indonesia has become resolute in favor of values truth. In the praxis level of life, people in Indonesia realize his responsibility to do what he knows is truth. The expression of truth is exquisitely transmitted in and through speech, attitudes, and his actions towards the natural environment, himself and others human. Character is a term that covers words, attitudes and actions that are consistent with the truth of religious teachings, customs, positive law, and does not conflict with universal human values. From this it can be understood if one of the important elements built in education is character. Even today, many of us are familiar with the term character education. So, it is not surprising that many students have raised character education as the title of the thesis considering that many of the nation's characters have faded. Second, people in Indonesia whose minds are advanced are intelligent cognition (know lots and lots to know) and his intelligence freed him from ignorance and delusion in various types and forms (for example: because colonialist engineering in the form of indoctrination). The term forward in mind shows increased intelligence and intelligence. Humans are advanced mind people who dare to think about the reality that bound their freedom, and dare to oppose against all forms of duping. Third, humans in Indonesia are progressing at a physical level or the body is not only physically healthy, but even more so has true knowledge of the functions of his body and understanding functions it is to liberate itself from all impulses towards crime. Humans who are advanced in aspects of the body are able to control encouragement-body demands. With and through that advanced body too, the advanced mind and the advanced character have support for declare self-independence from all forms of oppression of arrogant egos and greedy on the one hand and have the ability to assert self-existence civilized as a free human being (physically and spiritually) on the other hand. In the praxis of life, progress in the body can be understood as having the power to fight for independence and the skills to fill independence with all humanistic development (Haryati, 2019, p. 83).

On the third point, indirectly Ki Hadjar Dewantara asserted that education must produce a healthy generation physically and spiritually. First of all, by knowing the body's functions first, then he is being able to independently control his body. From this it can be taken a support that sex education should be empowered. This is because sex itself is something that has been inherent in humans from birth, and it grows naturally even unnoticed. Whether it's physical changes or psychological turmoil, both of them must be faced by everyone. Therefore, we should open our eyes to study this matter wisely. The meaning of the word sex itself sex, or matters relating to the genitals. In eastern culture, sex is privacy that must be hidden from public consumption. Making sex a taboo topic to be talked. Our culture can discuss sex as something sacred, but that does not mean it cannot be learned. The pattern of educating people who forbid sex to be bought with the assumption would be preferably shifted to the middle. In this case we need to review sex education that is appropriate for teenagers, not too vulgar and not too minimal in information. Furthermore, if the two words namely education and sex are combined, it will become a different term from the previous explanation. Some experts identify sex education according to their fields. For example, such as the opinion of Seto Mulyadi, et al., Who said that sex education is to instill an understanding of reproductive health and understanding of matters relating to sexuality issues in order to create a positive attitude towards sex (Tempo, 2020, p. 58). Whereas in an Islamic perspective sexual health education is defined as teaching, awareness, information about sexual problems that are given to the child since he was understand the problems which deals with sex, instinct, and marriage. In her study, Muhayati explained that Islam had early taught sex education. Examples include teaching the concept of shame through the limits of genitalia, instilling a spirit of masculinity or femininity through a conceptual identity in clothing, separating the parents' bed from the child, maintaining the cleanliness of the genitals, and the karma of the opposite sex. He also stressed the importance of the role of the family, especially parents through dialogical parenting. After family, school is the right place to teach sexual health education (Ubaidah, 2020, p. 123).

Based on the above explanations, it can be concluded that sex education is one of the efforts to instill the meaning of ideal sex to fit with good social values and religion. Not only that sex education must include physical, psychological, and social aspects. Sex education material must also be adjusted to the recipient whether it is children, adolescents, even adults. All of these things must be implemented optimally and cannot

be reduced so that it does not become a defective product. The explanation of sex education will be more complex when we question how important it is for the current situation. In addition to awareness of this we need to know how to achieve the objectives, as well as what methods are appropriate. All of these things will build a strong definition and in accordance with current conditions.

Cognitive Development Models and Environmental Influences on Adolescents Morale

Moral learning has been learned from generation to generation. Values and norms are instilled through the example of the behavior of the family and those around them. Then when children enter elementary school age, they learn to behave and socialize with their peers. They learn morals not only from the subject of manners, but through disciplinary habituation in their daily lives at school. Creating a conducive learning environment for them is not an easy thing. This is because morals must be integrated not only with certain subjects, but must be integrated into every element of education.

Regarding moral internalization, according to Kohlberg, human morals develop through three levels, and each level has two stages.

1. Pro conventional level

At this level, individuals view morals based on their own interests. That is, moral considerations are based on individual views regardless of the formulations and rules made by society. At this pro-conventional stage there are two stages as follows,

a. Obedience and Punishment Orientation
At this stage the child's behavior is based on the physical consequences that will occur. So, the child will avoid behaviors that lead to punishment. Therefore, every rule should lead to compliance to avoid negative consequences in the form of punishment.

b. Relative Instrumental Orientation
At this stage the child's behavior is based on a sense of fairness based on agreed upon rules. It is said to be fair when others repay every good deed. So, at this stage it is based on positive affirmations to strengthen the morals that have been worked on.

2. Conventional Level

At this stage the child approaches problems in individual relationships with society. The awareness in children begins to grow that the behavior must be in accordance with the norms and rules prevailing in society. In this way, problem solving is not only based on a sense of justice, but conformity with the norms of society. This level is divided into two as follows: Interpersonal Harmony: At this stage it is marked by each motivation from individual behavior driven on the willingness to meet the expectations of others. Individual awareness begins to grow that there are other people outside of him who will behave in accordance with his expectations. This indicates that the child begins to become aware of his relationship with other people that should not be broken, and Social System and Consciences: At this stage, individual behavior is no longer based on encouragement to meet the expectations of others who they respect, but is based on demands on society's expectations. This indicates that there has been a shift in awareness from oneself to social awareness. They have understood and accepted the existence of a social system that regulates individual behavior.

3. Conventional Post Level

At this level, behavior is not only based on compliance with the norms that apply to society, but on awareness in accordance with the values it has individually. At this level it is also divided into two stages, namely, Social Contract: At this stage, individual behavior is based on truths recognized by society. Individual awareness to behave grows because of the awareness to apply social principles. Thus, moral obligations are seen as social contracts that must be obeyed, not just value fulfillments. Universal ethical principles: Human behavior is based on universal principles. All kinds of actions are not only based on a social contract that must be obeyed but are based on an obligation as a human being. Every individual is obliged to help others. Motivation is not based on subjective reasons such as whether or not they like the person who is being helped, but prioritizes universal principles (Sanjaya, 2017, p. 243).

The cognitive development model presented by Kohlberg was inspired by Jean Piaget. He argued that human development occurs due to a process of cognitive restructuring which takes place gradually through certain stages. He explained how the relationship between moral, environmental, and individual cognitive development needs each other. However, based on his description, Kohlberg uses the basis of children's activities. Moral development does occur through social interaction. But this interaction has a special feature in which the child's personal factors come into play. Since this is to reach the highest possible stage of moral development, the child must be stimulated to be more active or passive. The point is more to hone children's abilities to be able to control what is in each individual. The essence of Kohlberg's theory is the emphasis on the importance of stimulation by the active effort of the individual to achieve his moral development, not passively imitating people without good reason. The right encouragement from adults will further stimulate children to determine the best attitude. With this the child will gradually develop his morals and then become part of his personality (Nurhayati, 2006, pp. 98–99).

The environment is the most flexible factor in the process of moral internalization. It is in the environment that there are sources of stimulation that affect child development. Even from the point of view of education, the creation of an effective learning environment to develop the potential of students is one of the goals. One educational environment is school, which according to Immanuel Kant is seen as a miniature of people's life. Another educational environment is the family which is the smallest unit of society. According to Santrock, sex education would be more appropriate if it was carried out in a family environment because it was included in the realm of privacy. However, if the family's capacity has not been fulfilled to do so, then school is an environment that needs to be pursued next. Of all the environments mentioned above, actually society is the key to successful moral development. Meanwhile, the family and school are unit units that carry out educational functions. After learning from school and family, teenagers will face the real field, namely society. It can be said that society is a place for teenagers to hone their adaptation skills, apply and study morals so that they are sustainable (Wahidah & Maemonah, 2020, pp. 30–31).

The next factor is the personality of the teenagers themselves. Kohlberg emphasized that there must be individual activity in an effort to develop his morals. Although the materials, educators, methods, and the same time were given to adolescents, the results were very likely to differ between adolescents. This is because every human being has unique and diverse characteristics. Some of the problems faced by adolescents today are due to the ideal image of adolescents should be and the ambivalent message to society in adolescents. Adolescent development researchers Shirley Feldman and Glenn Elliot the sexual message of society in adolescents is very ambiguous. Teens are expected to be sexually naive but have more sexual knowledge. Teenagers have to undertake this tough task in a society that is confused about how much and what kind of education to give teens. The next factor is the personality of the teenagers themselves. Kohlberg emphasized that there must be individual activity in an effort to develop his morals. Although the materials, educators, methods, and the same time were given to adolescents, the results were very likely to differ between adolescents. This is because every human being has unique and diverse characteristics. Some of the problems faced by adolescents today are due to the ideal image of adolescents should be and the ambivalent message of society in adolescents. Adolescent development researchers Shirley Feldman and Glenn Elliot the sexual message of society in adolescents is very ambiguous. Teens are expected to be sexually naive but have more sexual knowledge. Adolescents have to undertake this tough task in a society confused about how much and what kind of education should be given to adolescents (Nida, 2013, p. 273).

The development of adolescents is essentially built from three processes, namely biological processes, cognitive processes, and social processes. Cognitive processes include changes in an individual's mind, intelligence, and language. Memorizing, solving problems, imagining describes cognitive processes in adolescent development. Social emotional processes include changes in individual relationships with other humans in emotions, personality, and the role of the social context in development. Refuting parents, fighting with peers, the development of assertiveness, adolescent happiness in certain events reflect the role of social-emotional processes in adolescent development. The three processes are not separated or only partially carried out. This is because social processes shape cognitive processes, cognitive processes develop or inhibit social processes, and biological processes affect cognitive processes (Sholihah & Niam, 2020, pp. 22–23).

Psychosocial, Psychosexual Influence and the Role of Learning Environment on Adolescent Development

The rapid physical changes that occur continuously in adolescents cause them to be more aware of their body shape and try to compare it with their peers. If their perception is far from the reality of social standards, it will affect their psychological development. For example, girls tend to experience anxiety. In addition, parents also

often face conflicts when adolescents begin to grow, which is the root of the problem because it is difficult to understand their behavior which often invites uproar.

Based on the opinion of Eric Erikson, who is a psychodynamic theory follower, adds the theoretical basis of psychosocial development, in general, these stages of psychosocial development emphasize changes throughout the human life cycle. Each stage consists of a unique task that exposes the individual to a problem or crisis. If the individual is able to go through the crisis, the better the development will be. The eight stages of psychosocial development throughout the human life cycle are explained as follows:

1. Believe vs Don't Believe (0-1 year)

At this stage the baby has formed a sense of trust in someone. Usually, the people who are trusted for the first time are their parents or their careers. If there is failure at this stage, there will be distrust of others.

2. Autonomy vs Shame (1-3 years)

Children have started to try independently in motor tasks such as walking, climbing, or talking. This will work if in the process children are given a sense of autonomy to determine their behavior. However, most fail because parents tend to be overprotective of their children. This is what causes shame or insecurity.

3. Initiative vs guilt (3-6 years)

Children will take the initiative to find or do new things. They seek experience by relying on their sensory abilities. The final result obtained is the ability to produce something as an achievement. If the child is stopped or even prohibited, there will be feelings of guilt.

4. Perseverance vs inferiority complex (6-12 years)

Children will learn to cooperate and compete in academic activities or just socialize with peers. Children always try to achieve something they want so that persistence appears in them. If at this stage the child gets demands from his environment and the child fails to fulfill it, a sense of inferiority will arise. Therefore, reinforcement from parents or teachers is very important to strengthen children.

5. Identity vs Identity Confusion (12-20 years)

At this stage, there are many changes in a person, especially physical, as well as hormonal changes that will show their true identity. As well as self-orientation like where they will step in this life. All was done to find a new role and maturity status. Parents need to allow teens to explore these roles in different ways in each role. If adolescents go through this stage in a good way on a positive path to follow in life then a positive identity will be achieved. On the other hand, if a parent is forced on a role by their parents, and has not sufficiently explored the many roles, there is identity confusion.

6. Intimacy vs Isolation (20-40 years)

At this time individuals experience development by forming close relationships with other people. This describes intimacy by finding one's identity and at the same time losing the identity in others. When young adults form healthy friendships and close relationships with others, intimacy is achieved. If the circle of friends is unhealthy and detrimental there will be self-isolation. This is very dangerous because it can cause serious character problems. Young adults are born out of the search for and insistence on identity. They desire to merge their identity with the identity of others. On the other hand, intimacy is isolation, which means readiness to isolate oneself and if necessary, destroy the forces of people whose existence seems dangerous to them.

7. Generativity vs Stagnation (40-50 years)

At this stage the main concern is helping the younger generation to develop and direct life to be useful. This is called generativity. The feeling of being useless because he can't do anything to help the younger generation is called stagnation.

8. Integrity vs despair (60 years over)

In this stage a person reflects on the past and concludes that he has lived well or that he has lived a good life. In many ways, parents can develop a positive outlook at earlier stages of development. If this is the case, his retrospective glimpse will bring up a picture of life that can be put to good use, and satisfaction with the integrity he has achieved. Likewise, if the flashback of a person's life is negative then darkness or despair will appear (Erikson, 1994, pp. 73–80).

Another meaning of learning is to create an environment that enables the learning process, while the human being is both the subject and the object. Sex education is closely related to the process of adolescent development from all aspects, one of which is as mentioned by Erickson. Based on the understanding of adolescents in the previous chapter, the development experienced by adolescents is at the stage of Identity vs Identity Confusion, and Intimacy vs Isolation. The changes that occur in adolescents will show who he really is and what role he needs to take. All of these things are very much influenced by how the environment in which they develop, as well as how the character is in themselves. Recently there was a case of harassment committed by students at a university in Surabaya which was known as the Gilang's wrap case. This happens because their development fails to understand who their identity is, and there is self-isolation which leads to sexual disorientation. This is proof that sex education has become a necessity to implement.

Furthermore, the development of adolescents is studied from the psychosexual aspect. Sigmund Freud developed a psychosexual theory based on physiological maturity in certain parts or places on the body. Each developmental stage is characterized by the functioning of the libidinal impulses present in certain areas and which form the basis of all personality development with the characteristics of its behavior. This stage produces stages arranged in a fixed sequence and has a universal character in the human life cycle. In adolescents, psychosexual development occurs at the following 2 stages,

1. Latency Stage (6-12 years)

The period when sexual activity is calm, latent, and inactive. Although in some youth groups, sex is used as a topic of conversation in the form of jokes. The intensity is not as great as when the period before or after the latency period. It is also not personal, but a group. During this period there were indeed great, many, and multiple developments in all aspects such as cognitive development through formal education, social and moral development through extensive relationships with the environment. The period when children grow and develop basic skills, acquire and demonstrate a value system in their lives. He also learns the basics of being able to adjust to his social environment.

2. Genital Period (<12 years)

At a physiological point of view, maturity is the in particular the beginning to function of the genital glands. This affects the emergence of generous areas of the genitals as a source of pleasure and satisfaction. The sex drive in the real sense has emerged. The object of love changes from incestuous love to heterosexual love. This is a repetition and continuation of what happened in the mortal period. Therefore, it is likely that there will be a transfer of the object of love to the same sex. During this genital period there is a development in the direction of love. If previously his love was only one way, that is, centered on himself, now his love can be two-way. This is a sign of developing good adaptability in social relationships. Difficulties always arise by the existence of differences in norms, socio-cultural norms, and moral norms, both for the parents of adolescents and the surrounding community. These differences in norms often cause tension related to sexual problems in adolescents.

Furthermore, the learning environment that has the most influence is society. According to Vygotsky, development occurs in a social context. Children will be more affected by the people they interact with from birth. This made him think that culture has an important influence in learning. Culture is the determinant of the moral development of every human being. Each individual develops in the context of the culture that raised him. For example, the individual learning process is influenced by the main environment, namely the family. The cultural environment provides learning about what and how individuals think. Culture contributes to individual intellectual development in two ways, namely culture itself and the cultural environment. Through culture a lot of knowledge and moral values are obtained, then through the cultural environment it becomes a means of intellectual adaptation for individuals in the form of processes and means of thinking for individuals. Cognitive development results from a dialectical process by sharing learning experiences and problem solving with others. In this dialectical process, initially the individual takes responsibility for solving the problem, then through language he learns the vocabulary of knowledge in his culture. While developing, there is a process of internalization in the learning process. Internalizing knowledge is the first thing humans do in the dialectical

process. Then there will be a gap between what can be resolved on their own and what adults do, which is used as a standard. The source of learning is culture and the way adults solve problems. In fact, parents, neighbours, and peers are the most productive environments in their intellectual development. In his socio-cultural learning process, Vygotsky put forward four principles of learning. Among them are,

1. Social Learning, students learn through mutual interaction with adults or more capable friends.
2. Zone of Proximal Development, students will learn the concept well if they experience this stage. This means that if students are not able to solve their own problems, they need to be given assistance by more capable people. However, this assistance is only a guide, not a solution.
3. Cognitive Apprenticeship, which is a process that allows students to gradually acquire intellectual skills through interaction with more capable people.
4. Mediated Learning, namely giving scaffolding to students. Scaffolding is the provision of a large amount of assistance to students in the early stages of learning, then gradually reducing this assistance so that students have the opportunity to learn to be responsible for their problems. This will be very useful for him in the future to solve more complex problems.

In conclusion, the important point of all the explanations above is the essence of sociocultural learning. The interaction between internal and external aspects of the sociocultural environment is very important to consider in learning. According to Vygotsky, human cognitive function comes from the social interactions of each individual in a cultural context. He also believes that learning is most effective when the task is within the reach of students' cognitive abilities. For example, such as events or phenomena that often occur around him.

One of the efforts that can be applied to overcome these learning problems is the use of social constructivism-based learning tools. Social constructivism-based learning is learning that emphasizes students to construct their own knowledge in their minds by paying attention to social interactions. Researchers assume that the use of social constructivism-based learning tools is able to overcome the above problems. The arrangement of learning tools is aligned with the models, approaches and methods to be used in learning so that they are in a mutually supportive concept. This concept is used as the basis for researchers to apply the principles of social constructivism in learning tools. In this sense film is an appropriate form of social constructivism. This is because film is a medium that captures a phenomenon in society which is then used as a meaningful story. Through film, we can learn a variety of different perspectives on each member of society.

Results and Discussion

The Content of Sex Education and Hidden Moral Messages

More or less like that story "Dua Garis Biru" which lasted more than two hours. The reality in life portraits raised in this film, and there are also many moral messages related to sex education for teenagers. In addition, there are many analogies presented to make it easier for viewers to understand some taboos in the topic of sex education. Here are some analogies that appear in this film, as well as their meaning in relation to sex education:

1. The Seashells are open and closed

Figure 1. Scene in the film at 08:22 seconds

This illustrates the condition of the intimate areas of women whose conditions are similar to those of a virgin. First if the condition of the vaginal mouth is still tightly recognized as a virginity. This is the same as the condition of a closed clam which indicates that it is still fresh. Vice versa, when the condition of the mouth of the vagina is wide, it indicates that he has never had intercourse. This is the same as the state of open shells indicating it is not fresh.

The analogy relates to the notion of virginity in the opinion of most lay people. However, virginity is not actually judged by things like that. This was demonstrated when Bima made it clear to Dara that both fresh and non-scallops were good. This is what conveys the moral that a woman who is no longer a virgin is not determined by her hymen. Due to the mistaken community perspective on virginity, it makes victims of sexual abuse get bullied or other social pressures.

2. Strawberry

Figure 2. Scene in the film at 20:42 seconds

Providing explanations about reproduction to early adolescents can use an analogical approach to facilitate students' learning experiences. In the film it is explained that when the fetus begins to form, its size is like a strawberry. An explanation of abortion was politely packaged through the analogy of strawberries crushed in a blender. During this time an explanation of abortion sometimes shows real photos of abortion results. Although there are some who think this is effective in preventing the tendency of teenagers to get pregnant out of wedlock but it would be better if we convey it not by scaring.

3. Obstetricians

Figure 3. Scene in the film at 47:31 seconds

There are many roles as educators performed by obstetricians in this film. As an explanation of the risks of pregnancy at a young age, as well as about the test pack that was previously misunderstood by Bima. Here the role is very neutral and does not suppress Dara as a teenager who is pregnant out of wedlock. Instead, she gave an explanation of how to maintain a pregnancy to Bima and Dara. In addition, the author also quipped about the lack of sex education which only explains the reproductive system. This can be seen when obstetricians provide counseling to Bima and Dara in their practice room.

4. The Meaning of Dua Garis Biru

Dua Garis Biru is actually taken from the positive sign of pregnancy on the Test pack kit. But actually, on the Test pack tool the line color is pink. Meanwhile, the blue color is Bima's mistake in interpreting the Test pack. During an ultrasound examination of Dara's pregnancy, the doctor showed that their child was a boy. Then Bima denied it because on the Test pack tool Dara used the lines were pink. Bima also concluded that the pink color should represent the female gender, while the blue color for men. This teaches about the true use of the Test pack tool, and provides education about the importance of ultrasound for pregnancy checks.

Socio-Cultural Learning in Film "Dua Garis Biru"

There are many things that can be used as an afterthought in this film, especially about the importance of sex education being taught from an early age. Dua Garis Biru is a film that tells one of the social problems we know about marriage by accident. The ending of the story is also not clearly defined between a happy ending or a sad ending. However, what we can confirm is that the depiction of social reality is very clear in this film. As if insinuating everyone about our ignorance of the issue of sex that should be taught to teenagers.

As explained by Vygotsky, the learning process is more effective if it is based on its socio-culture. Teenagers in this film frame describe a lot of learning processes that come from their social life. Especially in this case, the influence of the environment does not only come from the real world, but also the virtual world. Like teenagers who accept the presence of the K-Pop trend that is endemic to teenagers, or teenagers who are still negotiating with their social environment. Even without a syllabus or curriculum, this film captures that teenagers learn more from what they pay attention to in their daily lives.

1. Reproduction posters in school student health units

There is a scene where Dara is forced to leave lessons to take a break at the UKS because of her pregnancy. There Dara cried because she felt the difficulty of being a pregnant woman. On the wall of the UKS there are posters about the structure of the reproductive organs of women and men. This seems to insinuate the lack of socialization about sex education which is only done through reproductive posters.

Figure 4. Scene in the film at 14:57 seconds

In addition, this film also insinuates about sex education material for adolescents, why only about reproduction? Meanwhile, regarding the consequences of sex in adolescence, the boundaries of sex that need to be understood, or the meaning of sex itself are still limited. This is what needs to be updated regarding sex education. In addition, this scene reminds us that sex education is a necessity for adolescents whose age is looking for identity.

2. School and The Scandal of The Pregnancy in Adolescent

Figure 5. Scene in the film at 33:47 seconds

Regarding the marriage by accident scandal, school policy should need to be reviewed. This film illustrates that the average school that finds students pregnant outside of wedlock will drop out without being able to defend it. In addition, this film also explains the truth about the reasons behind this policy only to protect the good name of the school, while the reality of the failure of education that has been implemented is ignored. This certainly has an impact on the future of students who are described as broken glass and cannot be repaired anymore. Therefore, this film actually contains a message to discuss again regarding what policies can help students who have made mistakes like this. The best punishment does not only have a deterrent effect, but also the awareness to become a better person. If this can be resolved without making students drop out, it will definitely not extend the case of underage marriage. In addition, mutual forgiveness needs to be taught from good school modeling. This is because cases like this must destroy students' self-concept so that they will experience obstacles in their psychosocial development. He will experience confusion regarding his identity, due to rejection from various parties. The worst impact of this is that adolescents who experience things like this will commit acts of hurting themselves or the fetus they are carrying.

3. Bridge of Life

Figure 6. Scene in the film at 42:09 seconds

Here is where Dara learns of life in one trip over the bridge. Described about the hustle and bustle of life that Dara, had never met when she was a child of a rich family. When the privileges that were inherent in her from birth have to be released to take responsibility for her pregnancy, she feels various emotions. Like when he has eye contact with a husband and wife who are fighting, or someone who dies makes him begin to understand that his life in the future will not be easy anymore. If it is understood that this scene actually teaches teenagers to see the reality of domestic life, it is not as easy as playing house. This should also be added to sex education for adolescents. They have to understand the big responsibility when they dare to cross the line, realities that are far from the fairy tales of kings and queens, so that they are able to create limits on themselves.

4. Ondel-Ondel and Parents

Ondel-ondel is a typical art of Jakarta in the form of a replica of the form of women and men. In ancient times, ondel-ondel was used to ward off evil spirits. Red-faced ondel-ondel is the embodiment of a man and fatherhood while the white-faced ondel-ondel is the embodiment of a woman and motherhood.

Figure 7. Scene in the film at 42:44 seconds

These ondel-ondel often appear when Dara or Bima are confused, worried and anxious. Except when they are eating shellfish, red ondel-ondel who want to enter the stall were expelled by the stall owner. Others appear when Bima borrows money from white-faced ondel-ondel for abortion. Dara also met white ondel-ondel when she was pondering wanting to make a decision. Both ondel-ondel illustrate the role of fathers and mothers who are never separated from children. Dara and Bima are teenagers who are forced to become parents early. The soul of a parent is actually always present in every individual, if it presents the courage to be responsible and the tenderness to love. Sex education should not only teach about what teens can or should not do, but also family education. To be a parent there are no special subjects at the formal school level, so the solution is for teens to imitate how parents are around them.

Figure 8. Scene in the film at 38:24 seconds

The dilemma of being a parent is indeed fairness. Even in this story, Dara and Bima's parents felt broken and failed. Anger has become one with sadness and confusion. Then forgiveness is an incomparable parent's magnanimity. When all parties dispose of and isolate Dara and Bima, it is the parents who grow their souls again through the door of forgiveness. Although not to understand their mistakes, but every mistake deserves forgiveness, and an opportunity to improve.

5. The reality of the harshness of domestic life

This film also tells how the real life after marriage. Pressure greater than the ability of the teenager cause them to experience a conflict. Bima, who has become Dara's husband, has to work hard to make a living. He works at Dara's father's restaurant as an ordinary employee. He had to do multiple things at one time. This is what makes it difficult following lessons at school, to make him skip class several times.

Figure 9. The scene in the film at 01:60:02 seconds

Besides that, Dara's conflict is no less complicated. Dara, who feels pregnant for the first time, sometimes has difficulty regulating emotions. Bima, who is truant, makes him worry about his son's future. Besides that, Dara is annoyed with Bima's childish behavior. He saw him having fun playing games on his cellphone. Bima's lack of attention makes him feel neglected. The two of them quarreled because they weren't able to understand each other's difficulties. This actually teaches teens who are busy with love about how important it is to prepare for a good future. In addition, there is an implied meaning of the amount of responsibility to be a parent. Conflict and

stress are very vulnerable to be experienced by every couple. Things like this should become common knowledge for teenagers. At least this will make teenagers think twice about doing things that are outside the boundaries such as sex before marriage. In addition, this film also depicts the reality of the emotional maturity of unstable adolescents. As explained by Erik, at adolescence there is a stage of looking for self-identity. This is marked by curiosity about what happened to him.

6. The Reality of Pregnancy in Adolescents

Most teenagers who have had sex before marriage, do it only to seek pleasure and satisfy curiosity. They haven't thought about the consequences such as earning a living or the hardships of bearing a pregnancy. As experienced by this pair of teenagers, namely Bima and Dara. After finding out about her pregnancy, Dara experienced many changes in her body. Then she searched the internet for all references about pregnancy. As described in the following scene,

Figure 10. Scene in the film at 1:13:20 seconds

In the scene, it is explained how Dara asks Bima to practice a tip before giving birth. Bima doubts it, because what they do is like a joke. Then Dara explained that she got the tips from the internet. From this we can understand that the current generation cannot be separated from the role of the internet. Meanwhile, the internet with millions of information is still lacking with good knowledge for us. Even pornographic content is widely spread on the internet. This is what we should fix in order to save teenagers from failing to understand about sex. Furthermore, the reality of pregnancy is also unknown to adolescents. They just imitate what adults do without knowing its purpose. Sometimes their body and mentality are not ready to endure pregnancy.

7. Social Criticism of the Family

Perversion of sex is considered a great disgrace that will lead to tremendous shame. The cynical looks and ridicule make the family feel a loss of self-esteem. But strangely, only Bima's family experienced criticism from his neighbors. Meanwhile Dara's family does not accept external conflicts, but rather internal conflicts between family members.

Figure 11. Scenes in the film at 53: 29-59: 03 seconds

After analyzing it, it turns out that what influences this is the environmental factor they live in. Bima, who lives in densely populated settlements and in the middle to lower class economy, is more prone to experiencing social criticism. The neighbors feel they have the right to ridicule him, because the culture that is created there is mutual cooperation. Meanwhile, Dara lives in a residential area that has more privacy. The environmental culture created by the neighbors is independent. So, they tend not to like taking care of other people's lives. This is evidenced in the scene at 53: 29-59: 03 seconds. There were discussions between Parents, and Bima as follows: Bima's Mother: *"Bima tidak usah nikah sama Dara? Kita ini tidak punya apa-apa,kita hanya punya iman dan harga diri. Mau jualan nasi udah malu, Pak. Ibu tau betul satu kampung ngomongin keluarga kita pak, Ibu tau. Kita ini udah gagal , gagal didik anak kita, Pak."* (Bima doesn't have to marry Dara? We don't have anything we only have faith and self-respect. I am embarrassed to sell rice, sir. I know very well that one village talks about our family, sir, you know. We have failed. failing our children, sir).

8. Early Marriage

Figure 12. Scene in the film at 1:03:04 seconds

Early marriage is still considered a solution for pregnant teenagers. This is considered the best solution, considering that children who are already in the womb need parental assistance. Bima and Dara also did this. This marriage was proposed by Bima's parents who have a religious background. Initially this was opposed by Mrs. Dara, who thought that her child was too early to marry. However, this marriage continued because almost all family members agreed.

Socio-Cultural Learning in Film "Dua Garis Biru"

Teenagers in the story are depicted well through their lifestyle and relationships. There are many scenes that show how teenagers live today. Like the bucin phenomenon that is sweeping the teenage world. They can already feel attraction to the opposite sex and even dare to have a relationship. It is not taboo for them when men and women make love to each other like adults. The youth depicted in this case are middle adolescents. They are adolescents who show the most vulnerable transition period. This is because adolescents are at the stage of forming characters by imitating role models. This is shown in the scene that shows Dara's hobby who likes K-Pop. Actually, it doesn't matter if teenagers have their respective hobbies. Even this hobby makes Dara have the ambition to continue her studies in Korea. This means that adolescents who have positive hobbies will make them have dreams. One thing that becomes a problem for adolescents is placing themselves to understand what boundaries need to be maintained, in this case related to moral aspects. The moral values of one nation with other nations must have differences. Coupled with the era of globalization that opens boundaries between countries, making it easier for teenagers to get to know other cultures, therefore, many teenagers now adopt which culture they like. Then imitate and learn all aspects related to hobbies or whatever they like. However, not all teenagers are like that. This is illustrated by the figure of Bima who grew up without an idol like Dara. He represents an ordinary teenager who has no ambition, but is quite good at respecting others. A typical teenager like Bima can be said to be an inferior group that supports a superior like Dara to grow. Uniquely, this film describes two different situations that are not common in our society. This explains to us that adolescents are now more open minded as evidenced by their respect for one another. Many things are not covered by our culture. Meanwhile, teenagers have experienced complex situations that require them to immediately find out. Like sex, the discussion of which is still very taboo in our culture, but things like that are an existing urge on adolescents.

The social norm in the form of exclusion is indeed a natural thing for society to do when assessing pregnancy outside of marriage. Pregnancy outside of marriage is considered a disgrace that can damage a family's reputation. This is like what happened to Big Sister Bima, namely Dewi. In this scene, Dewi hits and scolds Bima who has impregnated someone else's child. He explained about the circumstances that had to cancel the engagement due to Bima's behavior. He felt that what Bima had done could damage his family name in front of the community. This is because our eastern culture views adultery as a vicious act. Therefore, the perpetrator can experience exclusion, even his family can be ostracized. However, not everyone thinks that way. Sometimes there are some people who think this is normal. As the goddess said, he thinks his sister is stupid for having sex without using a condom. Actually, this sounds more like sarcasm about sex. The position on whether or not sex is legal in our society is ambiguous. This can be seen from how harsh the prohibition on sex is, but on the other hand the urge to know sex also occurs in adolescents. This suppresses adolescent growth because they have to experience a paradox about sex. At least this film invites the audience to re-discuss what good sex is like.

In this scene, Dara is confused because the milk seeps out, making her clothes wet. She was crying because she couldn't stop the milk from coming out of her breasts. He felt ashamed of his situation, especially when he was with his friends. Finally, she stuffed her breasts with tissue. Reality like this can actually be good knowledge for teenagers. The fact that pregnancy can change physical form, will definitely make teenagers more alert. This is because teenagers see their appearance as an obligation. Many teenagers are insecure about their bodies because they are different from the models in magazines. It can be said that appearance has an important meaning for adolescents.

In her psychosocial development, even though the teenagers have made mistakes in this film, she managed to find her identity. They realize the importance of viewing sex as something sacred. Even though they went through adolescence with mistakes, the process of recognizing their identities was successful. Like the scene where Dara's social contact returns when her friends visit her house. Dara became cheerful again after she was previously depressed because of her situation. With this social contact, at least Dara was able to restore her identity as a teenager.

Losing his identity as a child was restored by the role of his mother. Dara got her mother's attention and love back when she was sad because of her changing body. As we know, appearance is very important for teenagers. Therefore, Dara felt that she had lost her identity. The advice that came from his mother, restored the sense of worth that was in him. This is also experienced by Bima and his family. Bima was ashamed of what he had done. He also felt guilty for causing a lot of trouble for his family. As a boy, he also felt that he could solve this problem on his own, without bothering his parents. His mother also forgave and even apologized if all this time, he had not paid enough attention. Both of them forgive each other, and realize each other's mistakes. His mother also gave advice to Bima to become a responsible man. Bima's identity crisis was restored by the presence of his mother who gave him support and trust.

The Influence of Cognitive Development Models and the Environment on Adolescent Morals in Dua Garis Biru Film

Through its first debut in a trailer, the film Dua Garis Biru has drawn criticism for having a sensitive theme, namely sex. This has given a picture of our society which is still unfamiliar with sex itself. It is this view that has added to the length of the bad impact of the misunderstanding of sex itself. At least through this film, there are many depictions of the reality of society's depiction of sex. This can be used as a good reflection material for sex education itself.

Based on an interview from the author of “Dua Garis Biru”, it was explained that her motivation to create the “Dua Garis Biru” was because her concern for sex education in Indonesia was still minimal because it collided with society's views. The topic of sex is still considered taboo today. Therefore, “Dua Garis Biru” is here as a reminder for us to be ready for change and to open the taboo on sex, to find out how ideal sex education is for teenagers. Based on the Cognitive Development Model that occurs in adolescents in this film, it occurs gradually as follow,

1. Pro Conventional Level

Bima and Dara are teenagers who feel free to make their choices according to what they want. The first moral lessons they learn are when their test results are shared. Bima who got a bad score was immediately given a warning from his teacher. Meanwhile, Dara, who got a good score, defended Bima by saying that it was better

because it was done honestly. In the scene described forms the initial stage of his cognitive development of morals. First, orientation towards obedience and punishment when the teacher gives a warning to Bima, second, the relative instrumental orientation, when Dara, who has good grades, defends Bima by emphasizing that above good grades there is honesty as another good value. These two stages occur again when Bima and Dara are caught having free sex. The scene shown is when Bima and Dara are exercising. In the obedience and punishment orientation, Bima realized how big his mistake was when he saw the angry expressions of the parents and teachers. Bima realized that his mistake had to be corrected immediately. Then at the stage of relative instrumental orientation he also promises to be responsible for his actions. Obedience, and punishment orientation occur when Dara and Bima have to face the consequences of what they have done wrong. This forced the school to expel Dara from school, because her condition was pregnant. Meanwhile, Bima had to face social punishment in the form of exclusion provided by the social system.

2. Conventional Level

They thought about the punishment in the form of exclusion when Dara found out about her pregnancy. Therefore, Bima provides an abortion solution so that they can continue their normal life. But the truth came from her heart, Dara refused to have an abortion. Besides that, Dara also refused to give her child to others. Even though this contradicts her abilities as a teenager, Dara thinks that her child deserves the love of her biological parents. This stage is experienced by them when they start their life together after marriage. Bima, who began to realize that he had something to support, had to work hard until he decided to skip school. Meanwhile, Dara assesses the way the social system applies. He noticed that there was a tough task apart from providing for it, namely achieving his dream so that Bima could become a good father figure. This shows that a conflict occurs due to the absence of interpersonal harmony. They begin to understand how social systems work. Where the job of a father is obliged to provide a living, and a mother is obliged to look after her children. Many conflicts followed, especially those related to moral issues. Such as the consideration of how the child will be cared for considering the parents are still teenagers. In accordance with their conscience, Bima and Dara decided that Adam had to be cared for by one of his parents.

3. Post Conventional Level

At this stage both Dara and Bima understood how social contracts worked. Sometimes life doesn't go their way. The social contract works based on the morals adopted by the community. Although there are many differences, there is still a way to unite them. Like how important the role of a household head is. After giving birth, Dara was bleeding so she had to undergo a medical scenario for removal of the uterus. Bima was asked to make decisions about Dara's life. He had to choose between saving Dara's life but letting her live without a womb, or letting Dara fight her bleeding at the greatest risk of death. Finally, Bima chose the first option considering that life is more important. Furthermore, on universal ethical principles, there is a scene that touches blue. Between Bima and his mother, there was a deep conversation about how sorry they were in their hearts. Likewise, with Dara and her mother, they encourage each other to continue life.After their pregnancy got older, Dara and Bima realized how big the mistake they had made. Misperceptions about sex make them realize the importance of maintaining morals. This was shown when Bima and Dara both apologized to their parents. They realize that the prohibition of sex is actually done to protect their own good. No matter how big their child's mistakes, make their parents aware of universal ethical principles. Seeing regret makes them able to forgive.

Contextualization of Sex Health Education in Relation to Moral Internalization through the Film "Dua Garis Biru"

There are many values and concepts that are depicted in the film “Dua Garis Biru”. Through this film we realize how important sex education is because it is related to moral internalization. Sex education is one of the doors to instill morale in adolescents so that they are wise in making decisions for their future. The teenager is a confusing personality because he has to stand in the middle between being a child or an adult. As Erik Erickson said, at this time adolescence was trying to find his identity. This of course relates to aspects of its cognitive, biological, and social development. All three are factors of identity growth in adolescents. Often the biological factors in adolescents are ignored by considering the insignificance of the overflow of sexual development in them. The curious teenager's character gets him caught up in the wrong perception of sex. They identify it with pornography. Therefore, a bridge between the three is needed. The following is the contextualization of sex education in relation to moral internalization for adolescents.

Based on the description above, there are many things that should be built in sex education. This is because adolescents are active individuals who continue to learn through social portraits to measure what is on them. So far, sex education only emphasizes biological factors such as reproductive health. Meanwhile, consequences and moral internalization are not involved. So that the understanding of sex that occurs in adolescents is unable to ward off the risk of deviating from sex. Through the analysis of socio-cultural learning theory, we can understand that so far adolescents have studied sex from their surroundings. They study sex without filtering which information is correct. Therefore, teenagers understand sex by identifying it with pornography. Through psychosexual analysis we can understand that teenagers actually already have libido. This is what makes their understanding of attraction to the opposite sex accompanied by lust. However, this is often seen as something taboo to discuss with adults, even though emotional turmoil in adolescents does occur. Lack of answers from their situation, made them look for figures from other cultures. This is what underlies their actions to date as a middle way between avoiding negative views of society and the turmoil that exists within them. Teenagers are required to be sexually naive, but they are also required to be active in socializing with anyone.

Through psychosocial analysis, we can understand that adolescents are in the search for identity. Not only biological factors but social and cognitive factors should be considered. Adolescents who lack guidance on one factor will experience an identity crisis. This is what makes teenagers commit various kinds of delinquency. This should be overcome through moral internalization in order to form great character. Losing identity in adolescents will have an effect at a later stage. They may experience self-isolation rather than building social intimacy. Those who isolate themselves tend to experience problems at a later stage such as regret. This has a chilling domino effect on adolescents. Everyone's psychological endurance is different. If allowed to lose a significant stage in their life, it can lead to suicidal thoughts.

Through the analysis of cognitive development models, we can understand that adolescent's reason morally based on their cognitive development. The process of adolescent cognitive development is related to external factors such as the response of others to the actions they decide. There is a simple analogy that explains what morality really is through the trolley problem. It teaches that morality does not depend on how big the result is which is better, but rather an awareness to take action without guilt. If a teenager experiences an identity crisis, one of which is due to sex, then he will also experience moral conflict in his mind. They have many dilemma problems that require good moral understanding so as not to make mistakes. In this film, several dilemma problems are described, such as this teenage couple experiencing a dilemma when trying to get an abortion, then the option to dump their child to another parent. The decision to have an abortion or throw a child has a deep moral understanding. Often people think instantly that the mistakes they make must be immediately eliminated. They seldom consider the moral consequences that will have on their personality.

From the socio-cultural learning analysis, it can be applied that the context of sex has a lot to do with society. Understanding of the reasons for prohibition of sex in adolescence must be concerned with the cultural conditions that exist around him. Apart from that, understanding the stages in life is also teaching. For example, the reason why in adolescence is obliged to learn as much as possible. About the biological changes in adolescents should also pay attention. There are several differences in each individual that must be confused. Like the difference time you first get your period or the first experience of having wet dreams. All of these things need to be communicated openly from the youth side and addressed wisely by educators. This will form a relationship that understands and looks after each other. Besides that, the internalization of the noble values of our nation which adhere to Eastern customs can be implanted in the adolescence. Those who feel close and familiar with their culture will form the concept of identity in themselves. This will keep the culture sustainable and help youth pass through periods of transition comfortably.

Dua Garis Biru Film Media Framing as Sex Health Education Content and Moral Internalization for Adolescents

The problems raised in this film are of course related to sex and researchers see a connection with moral learning. This sex is related to the life of adolescents which is examined in two different perspectives. The first is the perspective that Bima has as the male main character. Second is the Dara's perspective as the main female character. The mistakes they made had a big impact on their next life. Like the domino effect, their mistakes creep up and even affect those around them. Teenage pregnancy is clearly considered a taboo subject to be appointed as content in a film. This is because sex outside marriage is considered adultery or a major sin in some societies, and others consider that such a situation is a barrier to the future. From these two different perspectives, both of them see sex in adolescence as something bad. This film provides various perspectives on how bad sex is as a teenager. In this film, sex is discussed in full using the point of view of several figures and

community groups. This results in many different points of view. This is due to the different backgrounds that are presented in the film. In addition, the character of each character is also a factor of difference from the point of view of sex. This makes sex into multiple interpretations. The following are stereotypical views of characters on sex:

1. Adolescents

In this group of adolescents represented by the main characters, namely Bima and Dara, while others are played by their school friends. Dua Garis Biru describe teenagers as a person who is up to date, trendy, close to social media, and also unstable. Regarding culture, they like products from other countries. As for the cultural products themselves, they are still less familiar with them. Like the scene of the ondel-ondel being chased away while they were eating. This of course affects the views of adolescents on their own cultural values. The taboo view of sex is a product of our society's culture. It was developed to protect adolescents from the bad effects of sex that are often identified with porn. Meanwhile, teenagers themselves experience tremendous turmoil that drives their curiosity about sex. Therefore, dating culture develops among adolescents. Dating is considered a middle way so that teenagers do not feel constrained because their curiosity about sex is limited by norms. Dating culture in adolescents often follows products from other countries. Like dating, physical touch, to having sex are already understood by teenagers. This makes the teenager's view of sex like something fun, not sacred. The teenagers depicted in this film show that they are able to understand the mistakes made by Bima and Dara. This shows that they are open minded but still careless because they do not understand the purpose of sex restrictions.

2. Family

There are two different backgrounds behind their differences in solving problems caused by wrong sex. The first family is from Bima who tends to take a religious approach with marriage to solve problems. Meanwhile, the second family, namely Dara's side, resolved the problem by handing over the baby in the child's stomach to the childless partner. So that Dara can return to focus on her goals. Both are contradictory, but their views on having sex before marriage, they agree that it is bad. The role of the family in this film shows the role of a problem solver for the mistakes of their children.

3. Society

The people who are most against free sex are society. They give a bad view of sex, as well as give social sanctions to the perpetrators. Here they ridicule and even isolate them and their families. Community behavior like this does have a psychological impact on the perpetrator and the family. The bad labeling that has been labeled by the community has a long impact on the social relations of the perpetrator and the family. This is what happened to Dewi, who was forced to cancel her engagement due to Bima's actions. She explained how embarrassed she would be if she had to meet her future husband's family. Of course, social sanctions like this continue to be carried out because they are considered effective in stopping casual sex. Although rules like this are unwritten, and not necessarily a negative stigma by everyone, Dewi's decision to cancel her engagement shows how strong the cultural roots of society are. Every member of society, especially adults understand very well about the sanctions against free sex.

From these three perspectives, it can be identified that the cause of the problem is the lack of discussion about sex. From the youth side, they are curious about what is happening to their bodies, and why they feel turbulent when they are with the opposite sex. Meanwhile, parents do not communicate with their children either because of work or high ego so that education about sex is neglected. In addition, parents are a member of society. The culture that develops in society is limiting adolescent access to sex because it is something that is taboo or inappropriate. This makes teens feel misunderstood and confused about how they respond to what happened to them. The ease of getting information from the internet encourages teenagers to explore sex in it. Meanwhile, in the internet world, the keyword sex is often identified with something that smells of pornography or eroticism. This is what encourages deviant sexual behavior in adolescents.

The moral assessment that is emphasized in this film is a feeling of regret that arises from sexual intercourse by adolescents. This regret is described in two perspectives. First, from the perspective of adolescents who are the perpetrators. Both parents are responsible for their children's behavior. In addition, this film also motivates everyone to rise from mistakes. They experience tremendous conflict until they understand how to put morals in order not to be left feeling guilty like before. The first conflict occurred after early marriage took place. Both Bima and Dara were unable to handle the conflict due to their lack of ability. This gives a moral message to teenagers why sex is strictly prohibited for them. The next conflict occurred when the two families debated the

fate of the child who was conceived by Dara. There is a solution to dumping their child to an expecting partner. This film conveys the moral value that no matter how bad people are, children are a gift that should not be hated just because they are born from a mistake. Every child has the right to be close to their parents.

The solution offered by the researcher is that this film can be used as a learning media. This can ward off the bad effects of misconceptions about sex. Given that there are many moral messages and various perspectives that are present in this film. This film has an ending that creates a dilemma. So, educators can use the discussion method. With discussion, we can find out how young people think and feel after watching the film. Then in addition it can be linked to Islamic values such as fiqh material about how the law of adultery is. In addition, the approach taken can use the learning theory developed by Vygotsky. Socio-cultural learning can provide an understanding for adolescents about how they should play a role in society. So far, teenagers live in their own world and don't mingle less with society. So, it is not surprising when adolescents do not know the culture, let alone the noble values upheld by society. Sexual behavior, although it seems trivial and dirty, in fact has a big impact on society. An area with a high degree of abuse against sex must have an effect on the moral level. The lower the moral level in society, the higher the crime rate, such as the killing of innocent babies, the increasing rate of abortion, the threat of life, and so on. Therefore, if people want to live side by side in peace, sex education should be encouraged.

Conclusion

A learning process always requires interaction, communication, message sources, message recipients, and the message itself. These messages are usually in the form of a code, symbol, picture, sound, or word. Through books, radio, videos, or films the recipient captures the message through their five senses and then processes it according to their cognitive development so that the message can be understood by the recipient of the message. The sign whether the message is accepted or not can be seen from the feedback given. To channel this message, learning media is needed. In the context of learning media is an important component for building communication between educators and students. Communication would be effective if both experienced events in the same area of experience. Therefore, the media is needed to deliver both of them to the same experience. Learning media can be classified based on learning experiences from the most concrete to the abstract. This is because the message implied in it is presented through the stories of other people being watched. This is because learning media is very closely related to techniques or learning systems, and is an inseparable part. Therefore, it is necessary to expand the meaning related to learning media so that instructors are more flexible in creating an effective learning environment. So, in this discussion, the meaning of the media taken is anything that can be used for learning. In other terms a film is also known as a moving picture. This is because a film is made of a series of images that slide quickly and are projected to give the impression of being alive and moving. The film is very impressive for anyone because it presents an audio, visual message. Even watching movies is currently a favorite activity for teenagers to spend time with their friends. Other advantages of films include, namely, providing messages that can be received more evenly to students, very good for explaining processes, overcoming space and time limitations, being more realistic because they can be repeated and stopped as needed, giving a deep impression so that they can influence student attitudes. However, films have other roles in the entertainment world which are closely related to the industry. This has an impact on the quality of films in Indonesia, which lack educational content. The culprit is only looking at the market of interest most people want. Therefore, it was not surprising that many scenes of romance, or fights were shown. The impact of the many films with minimal educational content has a major impact on the moral degradation of today's youth. At least the film can convey messages into the human soul more through the eyes and ears. Then it is able to make people remember the message as much as big even though it has only been watched once. So, it is only natural that people think what is conveyed through the film is a truth or an exemplary model. Most of the audience also did not filter the correct information because most of what was presented was the socio-cultural conditions that actually existed around them, but there was no resolution yet. Viewers will learn how the show will benefit them. Furthermore, if it is very impressive, it will affect the subconscious mind. People will have the same perception of the social environment that is on the spectacle. The social environment referred to here, includes film. How did this happen? This begins in the attention process to form perceptions. The attention process is a phase where the audience finds a sense of interest in a scene in the film. There are at least three things that attract the most potential attention, namely violence, sexism, and mysticism. After the attention is obtained, the perception is formed on the interpretation process which is also influenced by the cultural values possessed by the audience. Meanwhile, the truth value contained in the film is based on retention or repetition. The more often the film is repeated, the more likely it will influence the interpretation by confirming scenes in a film. Therefore, it is natural that violence, sexism, and myths are perceived as something that should be emulated, not something terrible anymore.

Recommendations

For educators: in order to save the future of the current generation, they must be brave to be open to discussing the theme of "sex" as an effort to cultivate morals. It is necessary to open up that affective learning should be taught by touching the hearts of students, not only teaching about understanding related to other cognitive matters. Nevertheless, it is necessary to seek an authentication of sex education that is appropriate to the Eastern cultural background adopted by most people in this country. This can be done by teaching sexual health education through the film "Dua Garis Biru" in order to form morale in students. This film should be taught with a discussion method by applying student active learning so that all messages can be captured into a good impression. For policy makers: researchers hope that sexual health education will become part of the educational curriculum. This needs to be done so that knowledge and learning media for sexual health education can be enriched even more. It needs a precise standard that can raise what sexual health education is appropriate to the cultural culture in this country. The goal is to form an integration between knowledge and real action of the nation's future generations. Then open up more research access and the realization of the concept of sex education is even better. For readers: to support the implementation of sexual health education for adolescents today. All the ideas and studies that have been carried out will not be realized if there is no change in the mindset of our society.

Scientific Ethics Declaration

As the authors of this work, we have ethical and scientific responsibility for its publication in EPHELS.

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References

Attwood, F., Barker, M. J., Boynton, P., & Hancock, J. (2015). Sense about sex: Media, sex advice, education and learning. *Sex Education*, *15*(5), 528–539.

Bowmaker, S. W., & Emerson, P. M. (2013). Still waiting for Mister Right? Asymmetric information, abortion laws and the timing of marriage. *Applied Economics*, *45*(22), 3151–3169.

Cameron, A., Smith, E., Mercer, N., & Sundstrom, B. (2020). 'It is our duty:' Understanding parents' perspectives on reproductive and sexual health education. *Sex Education*, *20*(5), 535–551.

Creswell, J. W. (2014). *Research design: qualitative, quantitative, and mixed methods approaches* (4th ed.). SAGE Publications.

Erikson, E. H. (1994). *Identity and the life cycle*. Norton & Company, Inc.

Eriyanto, E. (2001). *Analisis Wacana Yogjarkta*: Lkis

Ferguson, J. P., Xu, L., & Tytler, R. (2023). A pragmatist semiotic analysis of secondary students' embodied and material reasoning in astronomy. *Research in Science & Technological Education*, 1–24.

Formanek, R., & Gurian, A. (1980). *Why children ask?* Houghton Mifflin.

Gibs, J. (2012). *Mise-en-scène: Film style and ınterpretation*. Columbia University Press.

Haris, A. M. (2016). *Video as method*. Oxford University Press.

Haryati, H. (2019). *Pemikiran pendidikan ki Hadjar Dewantara: Studi tentang sistem among dalam proses pendidikan*. Uwais Inspirasi Indonesia.

Indrayani, W., & Jalil, A. (2016). Perilaku berpacaran pada remaja di desa batubelah kecamatan kampar kabupaten kampar. *Jurnal Online Mahasiswa (JOM) Bidang Ilmu Sosial Dan Ilmu Politik*, *3*(1), 1.

Kamal, S. M. M., & Ulas, E. (2022). Child marriage and its association with maternal health care services utilisation among women aged 20–29: A multi-country study in the South Asia region. *Journal of Obstetrics and Gynaecology*, *42*(5), 1186–1191.

Khoei, E. M., Kharaghani, R., Shakibazadeh, E., Faghihzadeh, S., Aghajani, N., Korte, J. E., & Esmkhani, M. (2022). Sexual health outcomes of PLISSIT-based counseling versus grouped sexuality education

among Iranian women with breast cancer: A randomized clinical trial. *Sexual and Relationship Therapy*, *37*(4), 557–568.
Lacković, N., & Olteanu, A. (2020). Rethinking educational theory and practice in times of visual media: Learning as image-concept integration. *Educational Philosophy and Theory*, *53*(6), 597–612.
Nida, F. L. K. (2013). Intervensi Teori Perkembangan moral Lawrence Kohlberg dalam dinamıka pendidikan karakter. *Edukasia : Jurnal Penelitian Pendidikan Islam*, *8*(2), 2.
Nurhayati, S. R. (2006). Telaah krıtıs terhadap teorı perkembangan moral lawrence kohlberg. *Paradigma*, *1*(2), 2.
Nurish, A. (2010). Women's same-sex relations in Indonesian pesantren. *Gender, Technology and Development*, *14*(2), 267–277.
O'Hara, M. (2010). Another ınconvenient truth and the developmental role for psychology in a threatened world*. *The Humanistic Psychologist*, *38*(2), 101–119.
Pfundmair, M., Eyssel, F., Graupmann, V., Frey, D., & Aydin, N. (2015). Wanna play? The role of self-construal when using gadgets to cope with ostracism. *Social Influence*, *10*(4), 221–235.
Sanjaya, W. (2017). *Paradigma baru mengajar*. Kencana.
Setiawan, A. A., Johan. (2018). *Metodologi penelitian kualitatif*. CV Jejak (Jejak Publisher).
Sholihah, D., & Niam, Z. W. (2020). Implications of moral education Lawrence Kohlberg and KH Ahmad Dahlan on the relıgıous behavıor of students. *Sunan Kalijaga International Journal on Islamic Educational Research*, *4*(1), 1.
Tangney, S. (2014). Student-centred learning: A humanist perspective. *Teaching in Higher Education*, *19*(3), 266–275.
Tempo, P. D. D. A. (2020). *Tokoh perlindungan anak dan perempuan bicara (seto mulyadi, erlinda, yohana susana yembise)*. Tempo Publishing.
Thompson, E. E., & Yeboah, A. A. (2013). Health information from elite to popular media: Are Ghanaian media creating more space for health information/education? *Critical Arts*, *27*(3), 370–385.
Trilling, D., & Jonkman, J. G. F. (2018). Scaling up content analysis. *Communication Methods and Measures*, *12*(2–3), 158–174.
Tyson, S. Y. (2011). Developmental and Ethnic ıssues experienced by emerging adult African American women related to developing a mature love relationship. *Issues in Mental Health Nursing*, *33*(1), 39–51.
Ubaidah, A. (2020). *Kesucian pendidikan seks dalam Islam.* Penerbit K-Media.
Vaisy, A. (2014). Sexual behaviors among Iranian women with cervical cancer. *Sexual Addiction & Compulsivity*, *21*(4), 263–275.
Wahidah, A. F. N., & Maemonah, M. (2020). Moral thought of early childhood in perspective lawrence Kohlberg. *Golden Age : Jurnal Pendidikan Anak Usia Dini*, *4*(1), 1.

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The Effect of L-Carnitine Supplementation on Sportive Performance

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Abstract: L-carnitine is a derivative of two essential amino acids synthesized in the liver and kidneys that our body needs for energy. In the bioenergetics of skeletal muscle, carnitine plays crucial roles. A severe reduction in muscular activity is associated with skeletal muscle carnitine insufficiency. Since L-carnitine supplement is known as a fat burner, its use among athletes has become more common in recent years. This review's objective is to assess the potential mechanistic effects of L-carnitine supplementation on athletic performance. In this context, the literature in the SportDiscus, EMBASE, PubMed and Google Scholar databases was scanned during a 15-year period to gather the data from the publications screened within the scope of SCI, SCI-Expanded, and ESCI. Consequently, it may be argued that taking carnitine supplementation improves athletic performance through a number of processes; including the maintenance of glycogen.

Keywords: Fat burner, L-carnitine, Exercise

Introduction

Dietary supplements are frequently used by competitors to preserve health and improve their performance in sports. There are many claims about L-carnitine, especially among sports supplements. Following rumors that it assisted the Italian national soccer team in winning the world championship in 1982, L-carnitine gained popularity. It is frequently referred to as a "fat burner" because it is said to increase the aerobic capacity by increasing fat oxidation and muscle mass while decreasing fat mass (Karlic & Lohninger, 2004). L-carnitine ß-hydroxy-gamma-trimethyl amino butyric acid is a derivative of two essential amino acids synthesized in the liver and kidneys that our body needs for energy (Mohammadi et al., 2016). Carnitine, synthesized from the amino acids lysine and methionine, has two forms: L-carnitine (biologically active) and D-carnitine (inactive). Carnitine is necessary for the transport of fatty acids to the mitochondria to be used in ATP synthesis. Since excess carnitine is stored in the body, it is always present in sufficient amounts in the muscles, and its deficiency is not possible (Roseiro & Santos, 2019).The largest source of L-carnitine in the body is found in skeletal muscles, where levels are typically around 50 and 200 fold greater than in the bloodstream (41 µM/L for females and 50 µM/L for males) (Ramsay et al., 2001). L-carnitine is also present in a variety of meals, although red meats like beef and lamb are the finest sources of carnitine. Besides meat, fish, poultry, and milk are also acceptable sources of carnitine (Pekala et al., 2011). Solely the bile and urine, where it has been shown that long-chain acyl derivatives congregate, are capable of excreting carnitine from the body (Ramsay et al., 2001).

Additionally, glycine propionyl-l-carnitine (GPLC), a new form of propionyl-l-carnitine (PLC) with diverse biological effects, has lately been used as a nutritional supplement (Mingorance et al., 2011). L-carnitine research in active, healthy populations has produced conflicting findings. More data supported by science are required to fully understand how L-carnitine can improve sportive performance (Koozehchian et al., 2018). For that, the aim of this review is to explain the mechanisms through which L-carnitine supplementation contributes to athletic performance.

Increasing Effect on the Use of Fatty Acids

Muscle metabolism uses mitochondrial fatty acid oxidation as a substantial source of energy, especially while performing exercise. Unfortunately, it seems like there is a limited amount of free L-carnitine available in the mitochondria throughout this process, particularly during intense activity. Thus, as exercise intensity increases from moderate to high, fatty-acid oxidation considerably declines. Given the significance of fatty acids in muscle bioenergetics and free carnitine's limiting effect on the oxidation of fatty acids during endurance exercise, supplementing with L-carnitine has been suggested as a way to enhance athletic performance (Muscella et al., 2020).

The main function of L-Carnitine is to transport long-chain fatty acids to the mitochondria for use in energy production. The cytosolic fatty acid form is long chain Acyl co-A (GovindShukla et al., 2019). L-carnitine acts as a substrate for the enzyme carnitine palmitoyltransferase and the production of acetylcarnitine, both of which are important for preserving a viable reserve of free coenzyme A (CoA) and sustaining flow through the tricarboxylic acid cycle (Wall et al., 2011). A group of enzymes known as carnitine acyltransferases (CATs) regulates the pool of acetyl-CoA and long-chain acyl-CoA in various sections, which is essential for maintaining energy homeostasis and fat metabolism (Colucci & Gandour,1988).Both the outer mitochondrial membrane (CPT I) and the matrix of mitochondria (CPT II) include carnitine palmitoyltransferases (CPT), which favor long chain acyl-CoAs as a type of substrate (Van der Leij et al., 2000). CPT1 converts long chain Acyl co-A to Acyl carnitine. Free L-carnitine is restricting the CPT1 reaction as a result of its buffering function (GovindShukla et al., 2019).

The passage of acyl carnitine across the mitochondrial inner membrane is mediated by mitochondrial translocase enzymes. Acyl Carnitine is converted into free carnitine and acyl-CoA by CPT2 (carnitine palmitoyl transferase II). Acyl-CoA undergoes beta oxidation, the resulting acetyl-CoA is included in the Krebs cycle. The energy is produced as a result of a series of reactions. Carnitine also helps convert pyruvate into citric acid in the mitochondria. It also regulates the activities of the pyruvate dehydrogenase enzyme and the adenine nucleotide translocase enzymes that control the ATP/ADP exchange within the mitochondria. Free carnitine then returns to the plasma and can react with a new long chain acyl-CoA. Based on this information, it has been reported that L-carnitine improves muscle endurance capacity by increasing fatty acid oxidation and energy consumption. (GovindShukla et al., 2019).

Glycogen Sparing Effect

The primary substrates for sustaining extended muscular contractions during endurance exercise are known to be carbohydrates and fat (Cermak & van Loon, 2013).Although carnitine is famous for its fat-burning properties, emerging evidence shows that it is also important in carbohydrate metabolism. It is clear that there is a strong correlation between muscle carnitine and the activity of the Krebs cycle, a very important in carbohydrate metabolism. The concentration of carnitine in muscle has been found to be directly proportional to muscle glycogen stores. Carnitine can act as a catabolic agent due to its 'glycogen sparing' effect to improve energy production from fats, effectively reducing the need to burn glycogen (Mojtaba et al., 2009). Simply put, a glycogen sparing effect makes more glycogen available later in physical activity, preserving plasma glucose availability and transport to the muscle to satisfy the CHO oxidation rates essential for maintaining the required activity (Jeukendrup et al., 1997). As a result of the first in vivo study**,** by modifying the acyl-CoA/CoA ratio, L-carnitine, regulates the activity of pyruvate dehydrogenase. Additionally, it inhibits the metabolism of carbohydrates, which eventually has the effect of sparing glycogen (Huelsmann et al., 1964).

Preventing Effect on Lactate Accumulation

L-carnitine is crucial for transferring long-chain fatty acids over the mitochondrial membrane and into the cells. Thus, it causes fatty acid oxidation to rise. High-intensity exercise can result in the production of acetyl-CoA and carnitine, which can combine to form acetyl L-carnitine and lower the acetyl-CoA to CoA ratio. It might lead to a reduction in the levels of lactate and a rise in pyruvate dehydrogenase activity. The transformation of acetyl-CoA to free CoA ratio rises during vigorous exercise, which causes more lactate to accumulate. Acetyl L-carnitine is created when L-carnitine and acetyl-CoA combine to adjust this proportion, resulting in less lactate buildup (Karlic & Lohninger, 2004).

Logically increased pyruvate dehydrogenase complex flux during intense activity would be anticipated to decrease blood lactate buildup, which may possibly protect glycogen stores and delay exhaustion (Spriet & Heigenhauser, 2002). Additionally, it's thought that L-carnitine could lessen lactate generation by preserving the

pyruvate dehydrogenase complex's enzymatic activity via a buffering process, lowering the acetyl-CoA/CoA balance (Barnett et al., 1994). Due to the steady pyruvate dehydrogenase complex flux and stable acetyl CoA/CoA ratio, L-carnitine has been found to minimize lactate buildup (Siliprandi et al., 1990).

Briefly, the main substrate in anaerobic activity is carbohydrate, and pyruvate is converted to lactate by lactate dehydrogenase, which affects sportive performance. The ratio of CoA to acetyl-CoA in mitochondria causes increased activity of the enzyme pyruvate dehydrogenase and eventually reduces lactate production. Carnitine increases pyruvate dehydrogenase activity and reduces lactate density, preventing fatigue (Arazi & Mehrtash, 2017).

Antioxidant Effect

The muscles in the skull are thought to have a major role in the oxidative stress brought on by exercise. This is while exercise increases the production of reactive oxygen species (ROS) and free radicals since movement is created by the skeletal muscles contracting continuously (Powers & Jackson, 2008). The primary factor contributing to the reduction in the NADH/NAD ratio in mitochondria appears to be contraction activity, which alters the redox state of muscles to one that is more oxidative (Sakellariou et al., 2014). Multiple ways that carnitine can support the body's antioxidant defenses. First, directly scavenging free radicals. It manifests this function by forming complexes with Fe+2, which accelerates the synthesis of reactive oxygen species, reducing iron levels and preventing lipid peroxidation (Powers & Jackson, 2008).

Second, by avoiding the creation of free radicals by blocking specific enzymes that produce free radicals or by preserving the integrity of the electron-transport chain in stressed mitochondria.The capacity of L-carnitine to control free radical-producing enzymes like NADPH oxidase, an oxidoreductase that produces ROS by transferring electrons from NADPH to molecular oxygen, is what gives it its antioxidant effects (Carillo et al., 2020).According to studies, L-carnitine inhibits Protein Hinase C (PKC) in a dose-dependent manner, which in turn transforms NADPH's phosphorylation state to that of a significant cytosolic component (Martins et al.,2002 ;Pignatelli et al., 2003).

Finally, by actively stimulating a variety of antioxidant enzymes and non-enzymatic antioxidants, mostly through transcription factors like Nrf2 and NF-B, to contribute to the preservation of the cell's ideal redox status.(Powers & Jackson, 2008). Currently, it has been established that the antioxidant activity of carnitine appears to be caused by the cellular control of antioxidant enzymes through the stimulation of the NF-B/IB system. According to an animal study examining the antioxidant effect of carnitine supplements, in the renal cortex of hypertensive rats compared to control rats, Nrf2, PPAR, and NF-kB expression were shown to be lower (0.3, 0.8, and 13-fold, respectively), but NF-kB expression was higher (Zambrano et al., 2013).

Method

The literature in the SportDiscus, EMBASE, PubMed and Google Scholar databases was scanned during a 15-year period to gather the data from the publications screened within the scope of SCI, SCI-Expanded, and ESCI for this review with ''l-carnitine''AND ''sportive performance supplements''AND ''carnite use'' keywords. The selected research were carefully reviewed, and an attempt was made to describe the ergogenic effects of L-Carnitine.

Conclusion

L-carnitine is a physiologically diverse component of an amino acid that comes in a variety of forms (Mingorance et al., 2011). In line with the research, it has been reported that carnitine supplementation can contribute to sports performance through mechanisms such as the maintenance of glycogen reserves, prevention of lactate buildup, increased antioxidant activity, and the utilization of fatty acids (GovindShukla et al., 2019). All of these factors suggest that -L-carnitine intake may have diverse impacts on various physiological and metabolic pathways, enhancing athletic performance during both medium and intense workouts (Pekala et al.,2016). To more fully examine L-carnitine's potential role in sportive performance, however, more research is necessary.

Recommendations

To begin with, even though L-carnitine is not technically a "banned" supplement, it is essential to confirm compliance with current WADA guidelines before a top athlete utilizes any supplement. The recommended L-carnitine dosage to improve exercise performance appears to be between **1 and 5 g**. L-Carnitine is non-toxic up to 15 g and has no side effects. Excessive amounts of L-carnitine (over 15 g) supplementation causes diarrhea. It has been reported that the bioavailability of L-carnitine is higher when taken naturally from foods compared to supplement form. Shortly, L-carnitine plays a role in energy synthesis and use; as such, it can be used as a sportive supplement for endurance during athletic competition.

Scientific Ethics Declaration

The author declares that the scientific ethical and legal responsibility of this article published in EPHELS journal belongs to the author

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References

Arazi, H., & Mehrtash, M. (2017). Effect of acute l-carnitine supplementation on blood lactate, glucose, aerobic and anaerobic performance in elite male artistic gymnasts. *Baltic Journal of Sport and Health Sciences*, *1*(104).

Barnett, C., Costill, D. L., Vukovich, M. D., Cole, K. J., Goodpaster, B. H., Trappe, S. W., & Fink, W. J. (1994). Effect of L-carnitine supplementation on muscle and blood carnitine content and lactate accumulation during high-intensity sprint cycling. *International journal of sport nutrition*, *4*(3), 280–288.

Carillo, M. R., Bertapelle, C., Scialò, F., Siervo, M., Spagnuolo, G., Simeone, M., Peluso, G., & Digilio, F. A. (2020). L-Carnitine in drosophila: a review. *Antioxidants*, *9*(12), 1310.

Cermak, N. M., & van Loon, L. J. (2013). The use of carbohydrates during exercise as an ergogenic aid. *Sports Medicine*, *43*, 1139-1155.

Colucci, W. J., & Gandour, R. D. (1988). Carnitine acetyltransferase: A review of its biology, enzymology, and bioorganic chemistry. *Bioorganic Chemistry*, *16*(3), 307-334.

GovindShukla Palkamshetti, A.,& Sampath Kumar, C.J. (2019). Carnilite capsules; helps to improve athletic performance & increase muscle recovery. *Pharmacreations,6(2),*19-23.

Huelsmann, W. C., Siliprandi, D., Ciman, M., & Siliprandi, N. (1964). Effect of carnıtıne on the oxıdatıon of alpha-oxoglutarate to succınate in the presence of acetoacetate or pyruvate. *Biochimica et Biophysica Acta*, *93*, 166–168.

Jeukendrup, A., Brouns, F., Wagenmakers, A. J., & Saris, W. H. (1997). Carbohydrate-electrolyte feedings improve 1 h time trial cycling performance. *International Journal of Sports Medicine*, *18*(2), 125–129.

Karlic, H., & Lohninger, A. (2004). Supplementation of L-carnitine in athletes: does it make sense?. *Nutrition*, *20*(7-8), 709–715.

Koozehchian, M. S., Daneshfar, A., Fallah, E., Agha-Alinejad, H., Samadi, M., Kaviani, M., Kaveh B, M., Jung, Y. P., Sablouei, M. H., Moradi, N., Earnest, C. P., Chandler, T. J., & Kreider, R. B. (2018). Effects of nine weeks L-Carnitine supplementation on exercise performance, anaerobic power, and exercise-induced oxidative stress in resistance-trained males. *Journal of Exercise Nutrition & Biochemistry*, *22*(4), 7–19.

Martins Chaves, M., Prates Rodrigues, A. L., Pereira dos Reis, A., Gerzstein, N. C., & Nogueira-Machado, J. A. (2002). Correlation between NADPH oxidase and protein kinase C in the ROS production by human granulocytes related to age. *Gerontology*, *48*(6), 354-359.

Mingorance, C., Rodríguez-Rodríguez, R., Justo, M. L., Alvarez de Sotomayor, M., & Herrera, M. D. (2011). Critical update for the clinical use of L-carnitine analogs in cardiometabolic disorders. *Vascular Health and Risk Management*, *7*, 169–176.

Mohammadi, M., Hajhossein Talasaz, A., & Alidoosti, M. (2016). Preventive effect of l-carnitine and its derivatives on endothelial dysfunction and platelet aggregation. *Clinical nutrition ESPEN*, *15*, 1–10.

Mojtaba, I., Maryam, C., Davood, K., & Fatemeh, K. (2009). The effect of chronic L-carnitine L-tartarate supplementation on glucose and lactate concentration and aerobic capacity. *Procedia-Social and Behavioral Sciences*, *1*(1), 2692-2695.

Muscella, A., Stefàno, E., Lunetti, P., Capobianco, L., & Marsigliante, S. (2020). The Regulation of Fat Metabolism During Aerobic Exercise. *Biomolecules*, *10*(12), 1699.

Pekala, J., Patkowska-Sokoła, B., Bodkowski, R., Jamroz, D., Nowakowski, P., Lochyński, S., & Librowski, T. (2011). L-carnitine--metabolic functions and meaning in humans life. *Current Drug Metabolism*, *12*(7), 667–678.

Pignatelli, P., Lenti, L., Sanguigni, V., Frati, G., Simeoni, I., Gazzaniga, P. P., ... & Violi, F. (2003). Carnitine inhibits arachidonic acid turnover, platelet function, and oxidative stress. *American Journal of Physiology-Heart and Circulatory Physiology*, *284*(1), 41-48.

Powers, S. K., & Jackson, M. J. (2008). Exercise-induced oxidative stress: cellular mechanisms and impact on muscle force production. *Physiological Reviews*, *88*(4), 1243-1276.

Ramsay, R. R., Gandour, R. D., & van der Leij, F. R. (2001). Molecular enzymology of carnitine transfer and transport. *Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology*, *1546*(1), 21-43.

Roseiro, L. C., & Santos, C. (2019). Carnitines (including l-carnitine, acetyl-carnitine, and proprionyl-carnitine). In *Nonvitamin and nonmineral nutritional supplements*. New York, NY: Academic Press.

Sakellariou, G. K., Jackson, M. J., & Vasilaki, A. (2014). Redefining the major contributors to superoxide production in contracting skeletal muscle. The role of NAD (P) H oxidases. *Free Radical Research*, *48*(1), 12-29.

Siliprandi, N., Di Lisa, F., Pieralisi, G., Ripari, P., Maccari, F., Menabo, R., Giamberardino, M. A., & Vecchiet, L. (1990). Metabolic changes induced by maximal exercise in human subjects following L-carnitine administration. *Biochimica et Biophysica Acta*, *1034*(1), 17–21.

Spriet, L. L., & Heigenhauser, G. J. (2002). Regulation of pyruvate dehydrogenase (PDH) activity in human skeletal muscle during exercise. *Exercise and Sport Sciences Reviews*, *30*(2), 91–95.

van der Leij, F. R., Huijkman, N. C., Boomsma, C., Kuipers, J. R., & Bartelds, B. (2000). Genomics of the human carnitine acyltransferase genes. *Molecular Genetics and Metabolism*, *71*(1-2), 139-153.

Wall, B. T., Stephens, F. B., Constantin-Teodosiu, D., Marimuthu, K., Macdonald, I. A., & Greenhaff, P. L. (2011). Chronic oral ingestion of L-carnitine and carbohydrate increases muscle carnitine content and alters muscle fuel metabolism during exercise in humans. *The Journal of Physiology*, *589*(4), 963–973.

Zambrano, S., Blanca, A. J., Ruiz-Armenta, M. V., Miguel-Carrasco, J. L., Revilla, E., Santa-María, C., Mate, A., & Vázquez, C. M. (2013). The renoprotective effect of L-carnitine in hypertensive rats is mediated by modulation of oxidative stress-related gene expression. *European Journal of Nutrition*, *52*(6), 1649–1659.

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Acute Effects of Myofascıal Release Exercıse Periods on Circulatory Parameters in Young Archers

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Abstract: The aim of this study was to investigate the acute effects of different myofascial release exercise durations on heart rate, saturation and blood pressure values in young archers. For this purpose, a total of 12 young athletes aged 16-18 years were included in the study. Myofascial release exercises using foam rollers were applied to the participants on different days. The inclusion criteria were determined as being healthy, not having a chronic disease, not having any medication used continuously and being interested in active archery sport. Control (T1), experimental (T2), and experimental (T3) foam rollers for 30 seconds and 60 seconds, respectively, were applied to the young archers on different days and heart rate, SpO2 levels and blood pressure values were measured after the applications. SPSS 22.0 programme was used to analyse the data obtained. One-way analysis of variance and LSD tests for repeated measures were performed to determine the difference between the groups of the application results. As a result of the analysis, no statistical significance was found in the saturation values, diastolic and systolic blood pressure values of the groups ($p>0.05$). There was statistical significance between T1 and T3 groups in the heart rate in favour of T3 ($p<0.05$). Myofascial release exercises affected the heart rate in terms of the parameters evaluated in long-term applications. It can be said that this change is a result of the energy and effort expended.

Keywords: Blood pressure, Exercise, Relaxation, Archery

Introduction

Archery stands out as a sport branch that has attracted the attention of mankind throughout history and has achieved great success today. This ancient sport attracts the attention of sports enthusiasts around the world with its advanced performance and technical skills, and also allows athletes to demonstrate their high level of physical and mental abilities. The essence of archery is a performance based on shooting arrows with high accuracy at a specified target within a limited time. This sport has a static character and requires intense strength and endurance of the upper body muscles. Archery includes the actions of traction technique, aiming and release techniques applied to the shooter in certain phases. The shooter carries the bow with the tense shooting arm with the muscles that keep the body posture stable during the shot and completes the movement by pulling the bowstring dynamically with the traction arm. After this moment, with the sound of the clicker, the archer completes and finishes the shot by releasing the bowstring (Behm et al.,2004; Fletcher & Jones, 2004; Fletcher & Anness, 2007; Gelen, 2017; Yonal & Turkmen, 2017; Aktepe, 2012).

This sport has a challenging process for top level athletes. Elite level archers have to maintain strong muscles and well-balanced upper body stability in this phase, which includes static and dynamic contractions. This process pushes the athletes to their limits, both physically and mentally, and encourages them to reach their best performance. In conclusion, archery, as both an ancient and contemporary sport, offers an experience that requires a combination of discipline, focus and technical skills. This sport has been recognised as a traditional

art throughout history and has maintained its value as a part of modern sport (Behm et al.,2004; Fletcher & Jones , 2004).

The warm-up process usually starts with aerobic running of increasing intensity starting at moderate levels. After moderate intensity, athletes move on to static stretching exercises. However, a number of studies have shown that warm-up movements at this level may negatively affect performance by decreasing the power, speed and force production of static and dynamic stretching exercises performed before, after training, before or after competition (Behm et al., 2004; Fletcher & Jones 2004). On the other hand, some researchers have reported that voluntary stimuli applied from moderate to high intensity, such as dynamic warm-up before sportive activity, may increase strength and performance by activating nerve-muscle function (Behm et al., 2006; Burkett et al., 2005; Fletcher & Anness, 2007; Gelen, 2017; Unlu et al., 2023). In the light of these data, the aim of this study was to investigate the acute effects of different myofascial release exercise durations on heart rate, oxygen saturation and blood pressure values in young archers.

Method

Participants and Study Design

Individuals who regularly participated in a private archery centre and attended training three days a week were included in the study. A total of 12 healthy male individuals between the ages of 16-18 years, who have been actively interested in archery for the last two years, were included in the study in accordance with the study criteria. The study criteria included being interested in archery for at least two years, participating in regular training, having no chronic disease, and not using any supplement or doping substance in the last six months. The researcher visited the subjects at their archery centres four times during the study period. In order to eliminate possible conditions such as physiological, neurophysiological and fatigue, the research protocol was applied to the subjects for three days with a 48-hour break. During the first visit, the participants were given a general information about the study and orientation sessions on the use of materials (foam rollers). In the second visit, age, height and weight measurements were taken and heart rate, saturation and blood pressure measurements were performed as a control practice. In the third visit, in addition to the control application, a 30-second session of myofascial muscle relaxation exercise with foam rollers was applied and measurements were taken. In the fourth and last visit, a 60-second session of myofascial muscle relaxation exercise with foam rollers was applied in addition to the control application and measurements were taken. To minimise the possible effects of circadian rhythm, all treatments were performed at the same time.

Implementation Procedure

All athletes were subjected to a general warm-up procedure before starting training and activities. The purpose of this warm-up was to prepare the body for physical activity, to flex the muscles and to improve performance. In this general warm-up phase, the athletes performed a 5-minute jog run, which raised their energy levels and increased their heart rate. Then, they performed 5 minutes of stretching and stretching movements to prepare their bodies for more mobility. These steps include measures to reduce the risk of possible injury by increasing the flexibility of the muscles. After the completion of the general warm-up, a special warm-up protocol was applied specifically for certain body parts. This protocol for upper and lower extremities aimed to maximise the athlete's preparation for certain movements by pre-activating the muscle groups to be used. In this way, it was aimed for the athletes to optimise their performance and benefit from the training in the best way (Unlu et al., 2023). In the control application, saturation measurements of the athletes were taken in resting and sitting position after general warm-up. Then, heart rate and blood pressure values were measured twice according to a specific test protocol. These controls aimed to carefully evaluate the general health status and physiological responses of the athletes.

Foam Roller Procedure

The foam roller exercises used in the research were preferred by focusing on various parts of the body, upper and lower extremities, posterior and anterior muscle groups in a way that the subjects could apply on their own. The participants applied rolling movements to the selected muscle groups using the foam roller and completed the movement by passing each muscle area from the starting point to the end point. The movement flow determined for each muscle group continued for 30 seconds and then a 20-second transition time was provided

to switch to another muscle group (Healey et al., 2014; Unlu et al., 2023). In this way, while each foam roller exercise continued for a certain period of time, the transition time between muscle groups was also provided in a certain order.

Statistical Analysis

Statistical analysis of the obtained data was performed using the SPSS 22.0 package programme (SPSS for Windows, version 22.0, SPSS Inc., Chicago, Illinois, USA). The analyses presented are expressed as means and standard deviations. The Shapiro-Wilk test was used to assess whether the data were normally distributed. One-way analysis of variance was applied to understand the distribution of the difference between repeated measurement data. LSD correction test statistic was used to determine the difference analyses between the treatments. Statistical significance levels were accepted as $p<0.05$. This value indicates that the results obtained are statistically significant.

Results and Discussion

This study aims to investigate the acute effects of myofascial release exercises using foam rollers. The study was conducted on a total of 12 volunteer athletes who participated in regular training with archery. Myofascial relaxation exercises were applied to the participants with different foam roller application times, and the effects of these exercises on heart rate, blood pressure and saturation levels were evaluated in detail. The data obtained during the research process were supported by tables and graphs, which are visual expression tools, and presented to the readers in a clear and understandable way.

Table 1. Presentation of the values of SPO2 levels

	Mean.±S.D	F	p
T1	96.33±0.89		
T2	96.50±2.11	1.222	0.309
T3	95.67±1.72		

Applications: T1. control treatment T2. experimental treatment 30 seconds T3. experimental treatment 60 seconds, *p<0.05

Table 2. Statistical analysis of heart rate

	Mean±S.D	F	p	Anlamlı Fark
T1	78.50±10.26			
T2	89.00±18.35	4.941	0.026*	T1-T3
T3	90.42±9.41			

Applications: T1. control treatment T2. experimental treatment 30 seconds T3. experimental treatment 60 seconds, *p<0.05

Table 3. Statistical analysis of systolic pressure values

	Mean±S.D	F	p
T1	11.50±0.67		
T2	11.50±1.24	0.478	0.585
T3	11.83±1.03		

Applications: T1. control treatment T2. experimental treatment 30 seconds T3. experimental treatment 60 seconds, *p<0.05

Table 4. Statistical analysis of diastolic pressure values

	Mean.±S.D	F	p
T1	7.50±0.67		
T2	7.42±0.67	0.865	0.421
T3	7.25±0.45		

Applications: T1. control treatment T2. experimental treatment 30 seconds T3. experimental treatment 60 seconds, *p<0.05

Conclusion

This study aims to evaluate the effects of various myofascial relaxation exercise durations performed with foam rollers used in archery sport on saturation, blood pressure and heart rate levels. The study was planned in

accordance with a controlled crossover experiment design. A total of 12 healthy male subjects between the ages of 18-20 years, who have been actively interested in archery for the last two years and who met the study criteria, participated in the study. The subjects were firstly subjected to the control exercise, then to the experimental exercise with foam rollers for 30 seconds and finally to the experimental exercise with foam rollers for 60 seconds. Afterwards, saturation, blood pressure and heart rate levels were measured and a detailed report was made. The main aim of this study was to determine the effects of different durations of myofascial release exercises with foam rollers, which are widely used in archers, on physiological parameters. Controlled crossover experimental design allows for more effective analysis of the data obtained and reliable interpretation of the results. In this context, it is of great importance to evaluate such exercises based on a scientific basis in order to optimise the performance of athletes and improve their post-training recovery processes.

In the results of the study, significance was found at $p<0.05$ level between control and experimental treatments in heart rate. No significance was found between the treatments in saturation and blood pressure values. Keeping the performance at a consistently high level in branches such as archery is a necessity that requires athletes to make intense efforts, to plan their training carefully and to be ready in every aspect. Competing in this sport tests not only physical abilities but also mental strength to a great extent. Athletes require great focus and discipline at every stage, from setting goals, starting training and performing in competitions. Therefore, an increased heart rate can be associated with this.

Nowadays, athletes have to constantly review their training strategies in order to maintain a high level of performance and outperform their competitors. This includes assessing various factors to increase their physical endurance, improve their technical skills and maintain their mental toughness. Training programmes should be customised to suit the individual needs of the athlete, as each athlete has a different performance potential and strengths/deficiencies. Especially in disciplines such as archery, focusing on the target and achieving a perfect shot requires athletes to constantly surpass themselves. This involves a constant endeavour to gain an edge over other athletes in competition. Competing against equally prepared opponents, getting better with each shot and constantly improving by trying different techniques are critical elements for success in the competitive world of archery (Healey et al., 2014; McGowan et al., 2015; Unlu et al., 2023; Yonal &Türkmen, 2017; Aktepe, 2012).

The use of foam rollers or foam roller applications in sports is recommended as an alternative method, which brings with it various advantages. The effects of these applications, such as reducing muscle soreness, delaying muscle fatigue, increasing joint range of motion and providing economy of movement, have the potential to improve the overall performance of athletes. Understanding how these advantages can be used in different sports can help to create optimised training programmes specific to the athlete's needs and discipline. In this context, research to understand how young athletes and amateur athletes can benefit from such applications can shed light on the creation of effective training strategies that appeal to a wide audience in sports. In addition, long-term evaluations of the performance-enhancing effects of foam roller applications may contribute to the determination of more effective training protocols for the long-term success of athletes (Chetham et al., 2015; Lim & Park, 2019; Unlu et al., 2023) . When we review the researches in the literature, we observe that foam roller applications are recommended for their ability to increase muscle function, optimise muscular efficiency, strengthen muscle strength, increase range of motion and improve flexibility. When this technique is used, it is thought that the friction caused by the rotational movement of the roller and the resulting increase in intramuscular heat contribute to improve the fluidity in the applied area. The results of the study show significance in all parameters evaluated. Therefore, we can say that acute foam roller exercises positively affect the performance of archers.

Recommendations

Conducting various applications in different sports branches can make a significant contribution to the scientific literature. At this point, studies on athletes of different age groups and genders can further enrich the knowledge base by enabling the data to be obtained to be spread over a wide perspective. In particular, studies focusing on the performances of female athletes in different disciplines can contribute to the understanding of gender-based differences in the field of sport.

Scientific Ethics Declaration

The authors declare that the scientific ethical and legal responsibility of this article published in EPHELS journal belongs to the authors.

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References

Aktepe K. (2012). *Okçuluk.* (1st ed.). Nobel Publishing

Axford, R. (1995). A*rchery anatomiy: An introduction to techniques for ımproved performance*. Souvenir Press Ltd.

Behm, D.G., Bambury, A., Cahill, F., & Power, K. (2004). Effect of acute static stretching on force, balance, reaction time, and movement time. *Med Sci Sports Exerc., 36*(8), 1397-1402.

Behm, D.G., Bradbury, E.E., Haynes, A.T., Hodder, J.N., Leonard, A.M., & Paddock NR. (2006). Flexibility is not related to stretch-induced deficits in force or power. *Journal of Sports Science and Medicine, 5*(1), 33-42.

Burkett, L.N., Phillips, W.T., & Ziuraitis, J. (2005). *The* best warm-up for the vertical jump in college-age athletic men. *Journal of Strength Cond Research, 19*(3), 673-676.

Cheatham, S.W., Kolber, M.J., Cain, M., & Lee, M. (2015). The effects of self-myofascıal release usıng a foam roll or roller massager on joınt range of motıon, muscle recovery, and performance: a systematic review. *Int J Sports Phys Ther. Nov, 10*(6), 827–838

Faigenbaum, A.D., Bellucci, M., Bernieri, A., Bakker, B., & Hoorens, K. (2005). Acute effects of different warm-up protocols on fitness performance in children. *Journal of Strength and Conditioning Research, 19*, 376-381.

Fletcher, I. M., & Anness, R. (2007). The acute effects of combined static and dynamic stretch protocols on fifty-meter sprint performance in track-and-field athletes. *The Journal of Strength & Conditioning Research, 21*(3), 784-787.

Fletcher, I. M., & Jones, B. (2004). The effect of different warm-up stretch protocols on 20 meter sprint performance in trained rugby union players. *The Journal of Strength & Conditioning Research*, *18*(4), 885-888.

Gelen, E. (2010). Acute effects of different warm-up methods on sprint, slalom dribbling, and penalty kick performance in soccer players. *TheJournal of Strength and Conditioning Research, 24*(4), 950-956.

Healey, K.C., Hatfield, D.L., Blanpied, P., Dorfman, L.R, & Riebe, D. (2014). The effects of myofascial release with foam rolling on performance. *The Journal of Strength & Conditioning Research*, *28*(1), 61-68.

Lim, J. H., & Park, C. B. (2019). The immediate effects of foam roller with vibration on hamstring flexibility and jump performance in healthy adults. *Journal of Exercise Rehabilitation*, *15*(1), 50.

McGowan, C. J., Pyne, D. B., Thompson, K. G., & Rattray, B. (2015). Warm-up strategies for sport and exercise: mechanisms and applications. *Sports Medicine, 45*, 1523-1546.

Unlu, M., Pancar, Z., & Karaca, B. (2023). The effect of different myofacial release exercise times using foam roller in archers on reaction balance and arrow shooting performance. *Gaziantep University, Journal of Sport Science, 8*(3), 250-258.

Yonal, G., & Turkmen, M. (2017). Turk kultur yasamında okculuk. *Akademik Sosyal Araştırmalar Dergisi*. 5(55), 523- 533.

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Age-Specific Reference Values for Amino Acid Content in Dried Blood Spots in Children in Western Kazakhstan, Measured by Tandem Mass Spectrometry

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Abstract: Measuring the level of amino acids in the blood is one of the stages in the early diagnosis of inborn errors of metabolism (IEM), implying timely initiation of therapeutic measures. Tandem mass spectrometry (MS/MS) is now replacing traditional IEM screening methods. Dried blood spot amino acid reference values developed for the pediatric population are crucial for interpreting test results and diagnosing aminoacidopathies. The study aims to establish reference values for amino acid (AAs) concentrations in samples of dried blood spots from newborns in Western Kazakhstan using LC-MS/MS (liquid chromatography-tandem mass spectrometry) technology. Methods: The cross-sectional study included 250 healthy newborns of Western Kazakhstan aged 1-3 days, born at term and breastfed, 49.2% male and 50.2% female. To establish the age-specific reference values for AAs, newborns were divided into three groups: (1) 1 day, (2) 2 days, and (3) 3 days. Blood samples on Guthrie cards were collected on days 1–3 of life and quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Nonparametric statistical approaches were used to generate the 2.5th–97.5th percentile distributions for newborns. Results: 3 of the 15 DBS amino acid distributions were dependent on gender. There was a statistically significant difference in the mean level of alanine, citrulline, and glutamic acid in males and females. The highest values were determined in the female group. Age-related differences in glutamic acid, leucine, ornithine, tyrosine, and valine concentration levels were observed. No significant correlations were found between the concentrations of 15 amino acids in dried blood spots and the body weight of newborns. Conclusion: The present study established amino acid concentrations that can be utilized as reference standards in Kazakhstan's newborn screening program for inherited metabolic diseases.

Keywords: Newborn screening, Amino acids, Dried blood spots, Tandem mass spectrometry Reference values.

Introduction

Measuring amino acid (AAs) levels in the blood is essential for the early diagnosis of inborn errors of metabolism (IEM), implying the timely commencement of therapeutic measures. The low incidence and prevalence of IEM cause them to be poorly studied and challenging to diagnose and treat (Céspedes et al., 2017). A significant problem with IEM is delayed diagnosis (5-10 years) or misdiagnosis due to the lack of specialized laboratories that perform accurate tests, resulting in delayed or lack of treatment. Early diagnosis of IEM can significantly reduce the risk of death and prevent long-term neurological complications (Scolamiero et al., 2015; Uaariyapanichkul et al., 2018).

Currently, tandem mass spectrometry (MS/MS) replaces traditional screening methods, which usually analyze individual biomarkers for each disease. This method can detect and quantify multiple IEM in a single blood spot

for most fatty acid oxidation disorders, aminoacidopathies, organic acid disorders, and urea cycle disorders (Mak et al., 2013; Uaariyapanichkul et al., 2018; Gelb et al., 2022).Dried blood spots (DBS) are usually the deposition of small volumes of capillary blood on special paper cards. Compared to whole blood or plasma samples, their advantages are that sample collection is more accessible, and there are no problems storing and transporting samples (Wagner et al.,2016). Reference values are crucial for interpreting test results and making a diagnosis (Lepage et al., 2006; Teodoro-Morrison et al., 2015; Adeli et al., 2017). Using carefully designed amino acid reference intervals appropriate for age, gender, and geographic location can facilitate the diagnosis of a wide range of disorders of physical, sexual, metabolic, and neurological development (Dietzen et al., 2016).

Pediatric populations require standards that reflect rapid physiological changes associated with growth, but these are often difficult to establish due to challenges related to obtaining sufficient samples from healthy children (Dietzen et al., 2016). Due to limited access to healthy controls in the pediatric population, laboratory data can provide information for estimating reference ranges (Dogan et al., 2017). New studies related to reference values of AAs in the blood of children are emerging all over the world, but most of them describe reference intervals for blood plasma (Yi et al., 2011; Méndez et al., 2013; Macit et al., 2014; Haschke-Becher et al., 2016).

In Kazakhstan, screening newborns using the MS/MS method is not mandatory, and there are no developed reference intervals for the concentration of AAs in DBS for different age groups of the pediatric population, including newborns. We initiated selective screening to obtain data on the frequency of IEM in children at risk in Western Kazakhstan. The results of selective screening tests in different age groups of the examined children should be interpreted by comparison with the reference values and/or threshold levels established for these groups. Therefore, one of the tasks is to establish reference intervals for the concentration of AAs in DBS in newborn children of Western Kazakhstan.

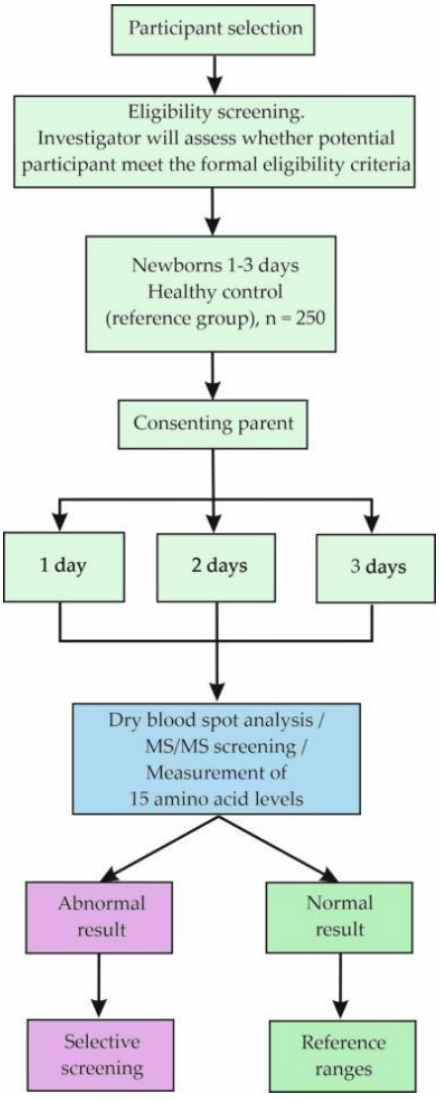

Figure 1. Study flowchart

Study Objectives

To establish reference values for AA concentrations in samples of DBS from newborns in Western Kazakhstan using LC-MS/MS (liquid chromatography-tandem mass spectrometry) technology.

Tasks

1. To set reference ranges of AA concentrations in samples of DBS of 250 newborns of Western Kazakhstan aged 1-3 days using LC-MS/MS technology.
2. To evaluate factors that may affect AA levels.
3. To compare findings of the determined analytes in newborns of Western Kazakhstan DBS with the results of previously published studies in other populations.

Methods

Data Sources

The data of this study were obtained during the examination of 250 healthy newborns aged 1-3 days to establish reference values of 15 AAs (Figure 1). The study was approved by the Bioethics Committee of the West Kazakhstan Marat Ospanov Medical University (Ref. No. 7, 09/09/2020.) Written informed consent (IS) was obtained from the parents and/or legal guardians of children after birth to collect a DBS sample. Demographic and anthropometric data of newborns are presented in Table 1.

Table 1. Demographic and anthropometric data of study participants

	Healthy newborns (n=250)			
	Group A 1 day (n=36)	Group B 2 days (n=116)	Group C 3 days (n=98)	The whole sample
Weight in grams, Median (IQR)	3434 (3180;3560)	3600 (3300;3833)	3615 (3470;3860)	3560 (3298;3830)
Gender				
Male, n, %	16 (44,4 %)	63 (54,3 %)	44 (44,9 %)	123 (49,2 %)
Female, n, %	20 (55,6 %)	53 (45,7 %)	54 (55,1 %)	127 (50,2 %)
Geographic distribution				
Urban population, n, %	22 (61,1 %)	68 (58,6 %)	54 (55,1 %)	144 (57,6 %)
Rural population, n, %	14 (38,9 %)	48 (41,4 %)	44 (44,9 %)	106 (42,4 %)

Criteria for Inclusion in the Study

Pediatricians examine all children in this study to ensure they do not suffer from any disorder or chronic disease. Healthy male and female newborns born after an uncomplicated pregnancy and vaginal delivery should have a body weight of 2500–4000 g, gestational age of 37–42 weeks, and an APGAR score greater than 7 in 10 minutes after birth. None should be diagnosed with birth asphyxia, defined as an Apgar score ≤6 at 5 min. All newborns must be breastfed, and their mothers must be healthy between 24 and 36. They must not have any food restrictions (vegetarian, vegan, etc.). Echograms of the placenta and fetus, as well as laboratory tests, should be normal throughout pregnancy.

Mass Spectrometry Analysis

Specimen Collection and Storage

Neonatal whole blood samples were collected from infants no earlier than 3 hours after feeding by heel prick using a heel stick. Five drops of whole blood (each ~75 µl) were applied to Guthrie cards, Ahlstrom 226 filter

paper, and PerkinElmer 226 Five-Spot Card (PerkinElmer Health Sciences, Greenville, USA) to form dried blood spots (DBSs) for LC-MS/MS analysis. Samples were dried for 4 hours at room temperature and then stored at 4°C in labeled individual zip-lock plastic envelopes with desiccants until analyzed by LC-MS/MS. Samples were sent to the laboratory within five days. In the case of long-term storage of samples, it was carried out at a temperature of –20°C.

Specimen Preparation and LC-MS/MS Analysis

The Neobase2 TM Non-derivatized MSMS kit (PerkinElmer, Wallac Oy, Turku, Finland) was used to quantify 15 amino acids in DBS according to the manufacturer's instructions. Vial with lyophilized isotope-labeled internal standards (IS) containing 2H3-Alanine (Ala IS), 2H4, 13C-Arginine (Arg IS), 2H2-Citrulline (Cit IS), 13C5-Glutamine (Gln IS), 13C5-Glutamic acid (Glu IS), 15N,2-13C-Glycine (Gly IS), 2H3-Leucine (Leu IS), 2H3-Isoleucine (Leu IS), 2H3-Hydroxyproline (Leu IS), 2H3-Methionine (Met IS), 2H6-Ornithine (Orn IS), 13C6-Phenylalanine (Phe IS), 13C5-Proline (Pro IS), 13C6-Tyrosine (Tyr IS), and 15N-13C5-Valine (Val IS) was being recovered by adding 1.4 ml of the extraction solution that is included in the Neobase 2 kit. The Extraction Working Solution (EWS) IS was prepared by diluting the recovered internal standards with the extraction solution of 1:100 (v/v).

DBS were analyzed using a Shimadzu LCMS-8050 Triple Quadrupole Mass Spectrometer (Shimadzu Corporation, Kyoto, Japan). Sample preparation was based on extraction followed by derivatization into oil esters. Level I and Level II (low standard and high standard) dried blood drops were included with each assay lot of the Neobase2 TM Non-derivatized MSMS kit to monitor system accuracy and precision.

To analyze amino acids and acylcarnitines, stored DBS card samples are brought to room temperature (+18 to +25°C) before extraction. A 3.2 mm disc (equivalent to ~3.1 µl of whole blood) is punched out of one dried blood spot with a diameter of 3.2 mm using a Wallac DBS Puncher (PerkinElmer, Wallac Oy, Mustionkatu 6, FI-20750 Turku, Finland) into the well of the 96-well polystyrene U-bottom microplate supplied with the Neobase2 TM Non-derivatized MSMS kit. After adding 125 µL of working extraction solution to each well of the microplate, the plate is covered with an adhesive aluminum film and incubated for 30 minutes at room temperature on a microplate shaker with a shaking speed of 650 rpm.

After incubation, 100 µL of the supernatant is transferred to a new 96-well U-bottom microplate, covered with aluminum foil to reduce evaporation, and incubated for 1 hour. The plate is then placed into the Shimadzu LCMS-8050 Triple Quadrupole Mass Spectrometer autosampler, and 5 µL of supernatant is injected into the LCMS for analysis.

Statistical Analysis

Shapiro-Wilk and Kolmogorov-Smirnov tests were used to check the normality of the distribution. The data obtained in the study demonstrated that the distribution of amino acids in DBS differs from normal. Me (median) and quartiles (IQR interquartile range) were used for descriptive statistics of the samples. Nonparametric tests (Mann-Whitney U test, Kruskal-Wallis H test) were used to test differences in AA concentrations depending on various factors (gender, age, place of residence).

Reference intervals in the group of healthy newborns aged 1-3 days were determined non-parametrically and corresponded to the 2.5-97.5th percentile of the experimental distribution. Considering the skewed distribution, correlations between body weight, age, and the concentration of amino acids in dry blood spots were performed using Spearman's test. Two-sided levels <0.05 are assumed to be statistically significant. Statistical analysis was done using the software IBM SPSS v. 23.0 (IBM, Armonk, NY, USA) and Statistica (StatSoft, Inc., Tulsa, OK, USA, v. 10).

Results and Discussion

Descriptive statistics and reference intervals for the concentration of 15AAs in whole blood of healthy newborns divided into subgroups according to age are presented in Table 2. For each analyte, the upper cut-off limit is set above the 97.5th percentile, while the lower limit is set below the 2.5th percentile.

Table 2. Amino acid levels in dried blood spots of 250 healthy newborns aged 1-3 days in Western Kazakhstan.

Amino acid, µmol/l		All children 1-3 days (n = 250)	Group A 1 day (n = 36)	Group B 2 days (n = 116)	Group C 3 days (n = 98)	Kruskal–Wallis *H* test	p-values
5-Oxo Pro	Median	86.68	99.23	86.68	83.83	1.26	0.532
	Range	53.89;108.87	69.90;108.87	46.51;111.34	58.57;107.40		
	2.5th-97.5th	22.70–159.87	22.64–68.93	22.24–165.99	22.70–156.05		
Ala	Median	265.01	270.11	254.66	285.48	2.46	0.292
	Range	213.91;315.71	199.07;301.89	211.54;306.94	223.70;325.32		
	2.5th-97.5th	146.27–422.57	137.55–371.65	161.94–461.45	146.27–422.58		
Arg	Median	18.13	18.46	17.02	19.17	8.96	0.013
	Range	15.56;22.68	15.85;22.73	14.12;21.65	16.07;22.68		
	2.5th-97.5th	11.63–33.14	12.45–33.01	10.42–32.94	13.11–35.33		
Cit	Median	19.56	18.51	19.13	19.58	0.591	0.744
	Range	15.97;22.65	16.54;22.39	15.55;23.32	16.83;22.41		
	2.5th-97.5th	11.76–30.54	11.64–26.25	11.76–30.54	12.41–31.15		
Gln	Median	411.97	415.11	417.74	402.29	1.95	0.376
	Range	352.17;441.54	374.88;490.63	385.32;501.08	317.02;496.21		
	2.5th-97.5th	201.24-648.33	208.75-678.42	215.30-685.40	189.13-674.83		
Glu	Median	428.80	477.05	390.92	438.17	15.74	0.0004
	Range	337.16;490.41	447.17;512.47	305.30;473.20	326.59;505.28		
	2.5th-97.5th	207.49–653.27	271.16–653.27	174.23–626.72	229.03–660.15		
Gly	Median	536.06	514.99	522.46	537.90	1.87	0.393
	Range	473.46;631.52	408.39;632.03	464.63;631.52	485.19;629.45		
	2.5th-97.5th	343.23–873.33	351.02–846.68	307.20–936.85	375.04–749.72		
Ile	Median	58.05	55.26	54.13	59.89	3.44	0.199
	Range	53.21;64.62	50.87;62.67	45.35;60.41	54.29;72.30		
	2.5th-97.5th	42.76 – 89.17	36.72 – 85.14	34.29 – 83.45	43.82 – 98.41		
Leu	Median	149.58	163.95	142.72	165.58	18.34	0.0001
	Range	129.44;178.26	135.48;171.08	125.03;162.33	136.12;196.07		
	2.5th-97.5th	96.04–231.24	114.04–219.71	88.24–225.64	115.90–257.35		
Met	Median	25.09	27.38	24.31	24.50	5.80	0.055
	Range	21.62;27.93	24.32;30.03	21.44;27.86	21.62;27.09		
	2.5th-97.5th	13.39–37.84	13.39–37,84	15.55–40.71	13.14–37.56		
Orn	Median	106.95	95.18	92.67	135.94	29.69	0.0000
	Range	80.98;144.38	76.90;130.03	73.00;117.92	105.48;162.91		
	2.5th-97.5th	49.31–205.45	49.31–166.99	45.81–226.01	66.42–205.45		
Phe	Median	60.44	62.82	60.62	58.82	3.11	0.212
	Range	52.98;67.63	50.99;70.78	54.21;70.17	50.90;66.71		
	2.5th-97.5th	43.22–92.43	43.83–92.43	40.79–108.62	43.36–85.37		
Pro	Median	159.15	145.79	160.72	157.35	1.18	0.553
	Range	139.75;185.77	136.44;191.58	140.50;182.36	139.75;192.37		
	2.5th-97.5th	106.43–246.79	115.25–228.15	103.25–260.13	106.43–246.79		
Tyr	Median	106.08	140.71	95.03	111.15	15.68	0.0004
	Range	81.93;141.62	97.90;164.04	78.62;122.72	84.83;139.68		

	2.5th-97.5th	52.53–248.17	58.72–298.39	37.78–252.27	54.99–205.94		
Val	Median	127.74	133.86	121.66	138.99	14.96	0.0006
	Range	107.47;151.18	101.68;154.18	100.95;141.93	115.70;161.42		
	2.5th-97.5th	85.41–194.93	80.51–187.07	71.50–195.81	98.22–194.93		

Differences in the distribution of amino acid levels in DBS between groups of newborns aged 1, 2, and 3 days, determined using the Kruskal-Wallis test, are recorded in Table 2. Statistically significant differences between age groups are noticed in the concentration of arginine, glutamic acid, leucine, ornithine, tyrosine, and valine (Table 2). In addition, significant weak positive correlations with age were established for the concentrations in DBS of leucine, ornithine, and valine, and weak negative correlations for methionine (Table 3).

Table 3. Statistical analysis according to age (Spearman's correlation) and gender (Mann-Whitney U test).

Analyte	Spearman correlation		Male N=123		Female N=127		p-values
	ρ	p-values	Median (μmol/L)	Range	Median (μmol/L)	Range	
5-Oxo Pro	-0.054	0.397	88.53	58.57;117.73	85.13	51.36;106.23	0.197
Ala	0.099	0.132	259.74	206.52;301.03	271.30	221.76;325.83	0.031
Arg	0.083	0.210	18.27	15.04;22.73	18.04	15.71;22.45	0.934
Cit	0.043	0.502	18.52	15.75;21.48	20.54	16.23;24.26	0,011
Gln	-0.023	0.668	413.74	362.55;459.57	408.10	321.17;435.49	0.502
Glu	-0.078	0.220	422.20	326.59;472.78	444.37	342.12;505.39	0.018
Gly	0.083	0.192	517.92	456.78;629.45	536.28	483.27;632.03	0.177
Ile	-0.041	0.305	61.44	58.08;68.07	56.30	47.58;60.31	0.121
Leu	0.173	0.007	155.48	129.46;184.30	146.71	127.61;171.08	0.128
Met	-0.126	0.047	24.62	21.04;27.57	25.69	22.22;29.13	0.071
Orn	0.304	0.000	105.69	81.83;149.17	108.79	79.09;143.02	0.845
Phe	-0.114	0.071	59.47	51.10;69.22	60.54	54.24;67.54	0.623
Pro	0.057	0.366	154.43	132.85;184.31	160.51	143.76;192.37	0.085
Tyr	-0.063	0.319	104.23	83.81;131.38	111.15	80.17;144.50	0.673
Val	0.189	0.003	128.92	104.06;148.56	125.82	109.37;154.93	0.849

Significant differences between female and male newborns were established by the concentration of alanine, citrulline, and glutamic acid in DBS (Table 3). In a study by Manta-Vogli et al. (2020), which assessed the concentration of amino acids involved in neurotransmission, statistically significant differences were also found between male and female newborns in the level of glutamic acid in DBS.

When assessing the effect of newborn body weight on the level of amino acids, no significant correlations were found between the concentration of 15 amino acids in dry blood spots and newborn body weight.

The levels of amino acids, as well as other metabolites in the blood, are influenced by several continuous and categorical variables, such as gestational age, birth weight, gender, ethnicity, age at blood collection, nutritional therapy (feeding pattern) and birth season, which have been shown to influence screening accuracy (Blanco et al., 2011; Ryckman et al., 2013; Hall et al., 2014; Clark et al., 2014; Peng et al., 2020). Environmental and nutrient changes over time, particularly protein intake, may affect amino acid levels in young children (Haschke-Becher et al., 2016).

We compared the results of measuring amino acid levels in DBS in newborns of western Kazakhstan with the findings of previously published studies in other populations (Dietzen et al., 2016; Haynes et al., 2016; Céspedes et al., 2017; Uaariyapanichkul et al. 2018; Yu et al., 2018; Sarker et al., 2019; Tan et al., 2021). Many researchers confirmed the relationship between gender and the level of certain AAs in DBS (Dietzen et al., 2016; Manta-Vogli et al., 2020; Uaariyapanichkul et al., 2018). The relationship between amino acid levels and birth weight was confirmed by Yu et al. (2018) and Manta-Vogli et al. (2020) but denied by Dietzen et al. (2016).

Conclusion

The present study established amino acid concentrations that can be applied as reference standards in Kazakhstan's newborn screening program for inherited metabolic diseases.

Scientific Ethics Declaration

The authors declare that the scientific ethical and legal responsibility of this article published in EPHELS Journal belongs to the authors.

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References

Adeli, K., Higgins, V., Trajcevski, K., & White-Al Habeeb, N. (2017). The Canadian laboratory initiative on pediatric reference intervals: A CALIPER white paper. *Critical Reviews in Clinical Laboratory Sciences*, *54*(6), 358–413.

Blanco, C. L., Gong, A. K., Green, B. K., Falck, A., Schoolfield, J., & Liechty, E. A. (2011). Early changes in plasma amino acid concentrations during aggressive nutritional therapy in extremely low birth weight infants. *The Journal of Pediatrics*, *158*(4), 543–548.

Céspedes, N., Valencia, A., Echeverry, C. A., Arce-Plata, M. I., Colón, C., Castiñeiras, D. E., Hurtado, P. M., Cocho, J. A., Herrera, S., & Arévalo-Herrera, M. (2017). Reference values of amino acids, acylcarnitines and succinylacetone by tandem mass spectrometry for use in newborn screening in southwest Colombia. *Colombia Medica (Cali, Colombia)*, *48*(3), 113–119.

Clark, R. H., Kelleher, A. S., Chace, D. H., & Spitzer, A. R. (2014). Gestational age and age at sampling influence metabolic profiles in premature infants. *Pediatrics*, *134*(1), e37–e46.

Dietzen, D. J., Bennett, M. J., Lo, S. F., Grey, V. L., & Jones, P. M. (2016). Dried blood spot reference intervals for steroids and amino acids in a neonatal cohort of the national children's study. *Clinical Chemistry*, *62*(12), 1658–1667.

Dogan, E., Uysal, S., Ozturk, Y., Arslan, N., & Coker, C. (2017) Selective screening for inborn errors of metabolism: a report of six years' experience. *Iranian Journal of Pediatrics*, *27*(5), e11323.

Gelb, M. H., Basheeruddin, K., Burlina, A., Chen, H. J., Chien, Y. H., Dizikes, G., Dorley, C., Giugliani, R., Hietala, A., Hong, X., Kao, S. M., Khaledi, H., Klug, T., Kubaski, F., Liao, H. C., Martin, M., Manning, A., Orsini, J., Peng, Y., Ranieri, E., …& Matern, D. (2022). Liquid chromatography-tandem mass spectrometry in newborn screening laboratories. *International Journal of Neonatal Screening*, *8*(4), 62.

Hall, P. L., Marquardt, G., McHugh, D. M., Currier, R. J., Tang, H., Stoway, S. D., & Rinaldo, P. (2014). Postanalytical tools improve performance of newborn screening by tandem mass spectrometry. *Genetics in Medicine: Official Journal of the American College of Medical Genetics*, *16*(12), 889–895.

Haschke-Becher, E., Kainz, A., & Bachmann, C. (2016). Reference values of amino acids and of common clinical chemistry in plasma of healthy infants aged 1 and 4 months. *Journal of Inherited Metabolic Disease*, *39*(1), 25–37.

Haynes, C. A., & De Jesús, V. R. (2016). Simultaneous quantitation of hexacosanoyl lysophosphatidylcholine, amino acids, acylcarnitines, and succinylacetone during FIA-ESI-MS/MS analysis of dried blood spot extracts for newborn screening. *Clinical Biochemistry*, *49*(1-2), 161–165.

Lepage, N., Li, D., Kavsak, P. A., Bamforth, F., Callahan, J., Dooley, K., & Potter, M. (2006). Incomplete pediatric reference intervals for the management of patients with inborn errors of metabolism. *Clinical Biochemistry*, *39*(6), 595–599.

Macit, E., Kizilgün, M., Çakir, E., Karaoğlu, A., Akgül, E. O., Oztosun, M., Aydin, I., Aydin, F. N., Agilli, M., Turker, T., Ogur, R., Gulcan Kurt, Y., Gul, H., Cayci, T., & Ozkan, E. (2014). Pediatric reference intervals for plasma and urine essential amino acids in a Turkish population. *Turkish Journal of Medical Sciences*, *44*(2), 323–329.

Mak, C. M., Lee, H. C., Chan, A. Y., & Lam, C. W. (2013). Inborn errors of metabolism and expanded newborn screening: review and update. *Critical Reviews in Clinical Laboratory Sciences*, *50*(6), 142–162.

Manta-Vogli, P. D., Schulpis, K. H., Loukas, Y. L., & Dotsikas, Y. (2020). Birth weight related blood concentrations of the neurotransmission amino acids glutamine plus glutamate, phenylalanine and

tyrosine in full-term breastfed infants perinatally. *Journal of Pediatric Endocrinology & Metabolism: JPEM*, *33*(3), 367–373.

Méndez, J. A., Fernández-Sanmamed, A. L., Gómez-Holgado, M. S., & Fernández-Rodríguez, F. (2013). Age-related reference values for plasma amino acids in a Spanish population measured by gas chromatography-mass spectrometry. *Journal of Pediatric Endocrinology & Metabolism: JPEM*, *26*(3-4), 333–341.

Peng, G., Tang, Y., Gandotra, N., Enns, G. M., Cowan, T. M., Zhao, H., & Scharfe, C. (2020). Ethnic variability in newborn metabolic screening markers associated with false-positive outcomes. *Journal of Inherited Metabolic Disease*, *43*(5), 934–943.

Ryckman, K. K., Berberich, S. L., Shchelochkov, O. A., Cook, D. E., & Murray, J. C. (2013). Clinical and environmental influences on metabolic biomarkers collected for newborn screening. *Clinical Biochemistry*, *46*(1-2), 133–138.

Sarker, S. K., Islam, M. T., Biswas, A., Bhuyan, G. S., Sultana, R., Sultana, N., Rakhshanda, S., Begum, M. N., Rahat, A., Yeasmin, S., Khanam, M., Saha, A. K., Noor, F. A., Sajib, A. A., Islam, A. B. M. M. K., Qadri, S. K., Shahidullah, M., Mannan, M. A., Muraduzzaman, A. K. M., Shirin, T., … Mannoor, K. (2019). Age-specific cut-off values of amino acids and acylcarnitines for diagnosis of inborn errors of metabolism using liquid chromatography tandem mass spectrometry. *BioMed Research International*, 3460902.

Scolamiero, E., Cozzolino, C., Albano, L., Ansalone, A., Caterino, M., Corbo, G., di Girolamo, M. G., Di Stefano, C., Durante, A., Franzese, G., Franzese, I., Gallo, G., Giliberti, P., Ingenito, L., Ippolito, G., Malamisura, B., Mazzeo, P., Norma, A., Ombrone, D., Parenti, G., … Ruoppolo, M. (2015). Targeted metabolomics in the expanded newborn screening for inborn errors of metabolism. *Molecular Biosystems*, *11*(6), 1525–1535.

Tan, J., Chen, D., Chang, R., Pan, L., Yang, J., Yuan, D., Huang, L., Yan, T., Ning, H., Wei, J., & Cai, R. (2021). Tandem mass spectrometry screening for inborn errors of metabolism in newborns and high-risk infants in Southern China: Disease spectrum and genetic characteristics in a Chinese population. *Frontiers in Genetics*, *12*, 631688.

Teodoro-Morrison, T., Kyriakopoulou, L., Chen, Y. K., Raizman, J. E., Bevilacqua, V., Chan, M. K., Wan, B., Yazdanpanah, M., Schulze, A., & Adeli, K. (2015). Dynamic biological changes in metabolic disease biomarkers in childhood and adolescence: A Caliper study of healthy community children. *Clinical Biochemistry*, *48*(13-14), 828–836.

Uaariyapanichkul, J., Chomtho, S., Suphapeetiporn, K., Shotelersuk, V., Punnahitananda, S., Chinjarernpan, P., & Suteerojntrakool, O. (2018). Age-related reference intervals for blood amino acids in thai pediatric population measured by liquid chromatography tandem mass spectrometry. *Journal of Nutrition and Metabolism*, 5124035.

Wagner, M., Tonoli, D., Varesio, E., & Hopfgartner, G. (2016). The use of mass spectrometry to analyze dried blood spots. *Mass Spectrometry Reviews*, *35*(3), 361–438.

Yi, P., Liu, L., Mei, H., Zeng, F., Huang, Z., & Niu, H. (2011). Establishment of reference range of plasma amino acids for younger Chinese children by reverse phase HPLC. *Journal of Pediatric Endocrinology & Metabolism: JPEM*, *24*(9-10), 733–738.

Yu, T., Han, Y., Pu, X., Duan, H., Zhi, L., Zhang, Y., Lei, L., Zhao, Y., Cao, Q., & Xu, H. (2018). Performance of LC/MS/MS in analyzing multiple amino acids and acylcarnitines in dried blood spot of very low birth weight, low birth weight, and normal weight neonates. *Clinical Laboratory*, *64*(9), 1333–1339.

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The Role of Tubulin and Thaumatin Genes and Osmotic Factors in Salinity Tolerance of Tomato Plants

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Abstract: Soil salinity is a drastic abiotic factor that affects many physiological processes and whole plants' activities, as well as up-and down-regulating gene expression. Studying the effect of salinity on tubulin and thaumatin relative gene expression as DNA markers for salinity stress in tomato plants is a scarcely studied topic. Thus, the present investigation was conducted to study the impact of different salinity levels (0, 75, 100, and 120 mM NaCl) on the tomato plants' growth, osmotic adjustment, and relative gene expression of both antioxidant and salt tolerance genes. As the salt concentration intensified, the fresh and dry weight of the shoots and the roots reduced significantly, accompanied by a reduction in chlorophyll a and carotenoids. On the other hand, salinity stress significantly decreased the level of osmotica (e.g., soluble sugars and soluble proteins) in tomato tissues compared to non-saline-grown plants, while a significant accumulation of free amino acids was recorded. At the molecular level, it was observed that the relative expression of the polyphenol oxidase, peroxidase, and thaumatin genes was high at the level of 100 mM NaCl, but it was suppressed at 120 mM NaCl. In contrast, salinity down-regulated tubulin gene expression in stressed tomato plants relative to controls, revealing various mechanisms that instigated salinity tolerance, which is concentration-dependent. The study recommended the importance of amino acids as osmotica as well as the relative expressions of PPO, peroxidase, and thaumatin genes in conferring salt tolerance at low to moderate salt levels.

Keywords: Salinity, Growth, Primary metabolites, Gene expression, Tomato

Introduction

Tomato (*Lycopersicon esculentum* Mill.) plants are one of the most frequently grown and consumed crops in the world. Egypt stands out for its tomato output, both for home use and export. It occupies the first rank among vegetables as a processed commodity in Egypt and many other countries (El-Khalifa et al., 2022). Salinity, or salt stress, which interferes with many phonological, physiological, biochemical, and molecular processes, is the second major abiotic factor lowering agricultural productivity worldwide. The tomato is considered to be a

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sensitive, tolerant, or moderately tolerant crop to salinity stress. In this regard, it has been reported that salinity stress lowered the germination percentage in the medium, fresh, and dry weight of the shoot, root length, chlorophyll a, b, and carotenoids contents, and K and Ca levels due to an increment in the tissue content of Na in tomato plants (Aazami, Rasouli, & Ebrahimzadeh, 2021). Many physiological attributes have been impacted by the damaging effects of salinity in tomato plants, including the increase of reactive oxygen species, membrane degradation, imbalance in ionic status, failure of the antioxidant system, deregulation of secondary metabolites, and hormonal imbalance (Dawood et al., 2022). However, the responses of antioxidant molecules or enzymes vary according to salinity level, exposure period, and plant growth stage (Parvin et al., 2019). The relationship between antioxidant activity and salt tolerance development may be associated with some alterations in their gene expression. As a defense mechanism against the harmful effects of salt, plants accumulate metabolic products, such as sugars, proteins, and proline as osmotica, to up-regulate the osmotic status of tissues (Sheteiwy et al., 2022). Furthermore, at the molecular level, plants tolerate salinity stress by activating stress-related genes (Raza et al., 2022). At present, many functional genes for protecting cells have been characterized, including heat shock protein (HSP), late embryogenesis-abundant (LEA) protein, antioxidant enzymes, and membrane transporters (Liu et al., 2023). Genetic engineering of novel stress-tolerance-related genes from plants in special habitats can provide candidate genes for cultivating crop varieties that can tolerate abiotic stresses. The overexpression of specific stress response genes in plants capable of surviving in each stress environment is evoked as a common adaptive mechanism. To conserve plant cells from oxidative damage induced by abiotic stresses, such as salt stress, the organization of several genes encoding antioxidant enzymes could take into account their payoff in the salt stress response in plants, such as plant peroxidases and polyphenol oxidases (Azzam et al., 2021).The molecular mechanisms of changing cell wall dynamics are highly affected by salt stress. The tubulin proteins are the molecular building blocks of microtubules, which have a remarkably maintained call shape via conserving the structure of the cell wall (Janke & Magiera, 2020). However, the mechanisms of changing cell wall dynamics through salt stress and cell wall integrity pathways remain unclear (Chun et al., 2021). Thaumatin-like proteins (TLPs) are the products of a huge, very complicated gene family that participates in host defense and a variety of developmental processes in fungi, plants, and animals. The structural diversity of TLPs in plants is connected to a wide range of traits. The majority of their anticipated activities are in relation to biotic challenges, whereas others are in response to abiotic conditions, including drought and osmotic stress. Yet, the roles of certain TLPs superfamily members have not been fully established (de Jesús-Pires et al., 2020). The quality and quantity of crops are influenced by both biotic and abiotic factors; as a result, this research aims to: i) investigate the influence of salinity as a common problem in the Egyptian soil on the osmoprotectants ii) detect antioxidant-related defense gene expression, and iii) study the behavior of tubulin and thaumatin gene expression in tomatoes under salinity stress.

Material and Methods

Experimental Design

A pot experiment was undertaken in the greenhouse of the City of Scientific Research and Technological Application (SRTA-City), Borg El-Arab, Alexandrina, Egypt, under natural conditions of humidity, temperature, and light during the year 2022. Seedlings of the tomato hybrid T-186 (45 days old) were transplanted into the experimental pots. Each pot was lined with a plastic sac and filled with one kilogram of soil (1 clay: 2 sandy). Four different concentrations of salinity (sodium chloride) were prepared at four levels: 0, 75, 100, and 120 mM. Five pots per treatment were applied. The plants were irrigated with tap water on a field capacity (40%) basis. Tomatoes were kept under these conditions until harvested after 30 days for phonological, physiological, and gene expression studies.

Plant Analysis

Growth Parameters

Three replicated plants per treatment were sampled for growth measurements. At harvest, roots and shoots were immediately separated, washed with distilled water to remove any additional salt surface contamination, and dried on absorbent paper. Fresh weights were directly recorded, and samples for dry weight determination were taken.

Determination of Photosynthetic Pigments

The fractions of pigments (chlorophyll a, chlorophyll b, and carotenoids) were estimated using the spectrophotometric method recommended by Lichtenthaler (Lichtenthaler, 1987).

Determination of Osmolytes Content

Soluble carbohydrates were measured based on the anthrone sulfuric acid method. (Fales, 1951; Schlegel, 1956). According to the method of Lowery et al., soluble proteins were determined (Lowry, Rosebrough, Farr, & Randall, 1951). Ninhydrin assays were utilized to examine free amino acids (Lee & Takahashi, 1966). Soluble carbohydrates, soluble proteins, and free amino acids content were estimated by the preparation of calibration curves using pure glucose, bovine serum albumin, and glycine, respectively.

Fourier Transmission İnfrared Spectroscopy (FTIR) of Tomatoes' Leaf Contents

The Fourier transform infrared (FTIR) spectra for dry-grinded tomato leaves were recorded using a Nicolet iS10 (Thermo Scientific, USA) with 1 cm^{-1} resolution and a range of 500–4000 cm^{-1}. Furthermore, the KBr-Wafer method was used (Hsu & Lo, 1999).

Relative Gene Expression of POD, PPO, Thumatin, and Tubulin Using Quantitative RT-PCR (qRT-PCR)

Extraction of RNA and Synthesis of cDNA from Tomato Plants

One hundred milligrams of tomato leaves were used for total RNA extraction by the TRIzole LS Reagent Kit (Chomczynski & Sacchi, 2006). The purity and concentration of extracted RNA were measured by Nanodrop Thermo Scientific, model 2000c, USA, at A260/A280. In order to synthesize cDNA, 1 µg of DNase I-treated RNA was utilized from each sample as a template in a reverse transcription process. The reaction procedure was carried out with oligo (dT) and random hexamer primers, and the reaction conditions and components were applied in accordance with previously established protocols (Abdelkhalek et al., 2019). The final cDNA product was kept at -20 °C until utilized as a template for qRT-PCR.

Expression of Defense Genes in Treated and Non-Treated Tomato Plants

Quantitative RT-PCR was used to measure the transcript levels of five tomato genes (peroxidase, polyphenol oxidase, thaumatin (TLPs), and tubulin (TUB)) and β-actin for all regimens. Table 1 displays the nucleotide sequences of the primers used. The expression levels of the PPO, POD, TUB, and TLPs tomato genes were adjusted using the β-actin gene as a standard. Each biological treatment's qPCR reactions were conducted in a separate run using a SYBR Green Mix (Thermo Fisher, CA, USA) and a Rotor-Gene 6000 real-time thermocycler (QIAGEN, Germantown, MD, USA). The PCR reaction mixture and the reaction conditions were performed as reported in a previous study (Hafez et al., 2013). Using the $2^{-\Delta\Delta CT}$ method, relative expression levels for each studied gene were estimated (Livak & Schmittgen, 2001).

Table 1. The nucleotide sequences of the primers used in this study.

Primer and Gene Name	Abbreviation	Direction	Nucleotide Sequence 5`-`3
Peroxidase	POD	Forward	GCTTTGTCAGGGGTTGTG AT
		Reverse	TGCATCTCTAGCAACCAA CG
Polyphenol oxidase	PPO	Forward	CATGCTCTTGATGAGGC GTA
		Reverse	CCATCTATGGAACGGGAAGA
Thaumatin	TLPs	Forward	CATGTCCTCCCACAGAGTAC
		Reverse	ATATAATCCCATTTCGTGCTTATG
Tubulin	TUB	Forward	AGGATGCTACAGCCGATGAG
		Reverse	GCCGAAGAACTGACGAGAATC
β-actin	β-actin	Forward	TGGCATACAAAGACAGGACAGCCT
		Reverse	ACTCAATCCCAAGGCCAACAGAGA

Statistical Analyses

Using the SPSS 21 software, all data were statistically analyzed using a one-way ANOVA. Tukey's honest significant differences (H.S.D.) at a probability value ($p \leq 0.05$) were applied to the obtained data, where three replicates were applied per treatment.

Experimental Results

The different concentrations of NaCl salinity significantly impacted the fresh and dry weights of shoots and roots. As represented in Table 2, salinity stress caused a reduction in shoot fresh weight (SFW) by 10%, 15%, and 17%, as well as 31%, 55%, and 53% for root fresh weight (RFW) at the levels 75, 100, and 120 mM, respectively, compared to control. Also, the dry weight of shoots and roots was significantly reduced by salinity stress, where the percent reduction of shoots (SDW) was 7%, 30%, as well as 50% and 48% for root dry weight (RDW) at the levels of 100 and 120 mM, respectively.

Table 2. The fresh and dry weight of shoots and roots of tomato plants grown under different levels of NaCl salinity stress (0, 75, 100, and 120 mM); SFW = shoot fresh weight; RFW = root fresh weight; SDW = shoot dry weight; RDW = root dry weight. The significant difference is ** at $p \leq 0.01$ and * at $p \leq 0.05$.

NaCl Treatments	Biomass			
	SFW	RFW	SDW	RDW
0 mM	$5.93^{b} \pm 0.26$	$4.30^{b} \pm 0.52$	$0.95^{a} \pm 0.13$	$0.4^{b} \pm 0.13$
75 mM	$5.32^{ab} \pm 0.1$	$2.95^{a} \pm 0.03$	$1.27^{ab} \pm 0.19$	$0.49^{b} \pm 0.07$
100 mM	$5.05^{a} \pm 0.09$	$1.93^{a} \pm 0.19$	$0.88^{ab} \pm 0.03$	$0.20^{a} \pm 0.02$
120 mM	$4.93^{a} \pm 0.15$	$2.00^{a} \pm 0.17$	$0.66^{b} \pm 0.08$	$0.21^{a} \pm 0.01$
F value	7.48*	14.59**	4.07*	12.19**

As was expected, soil salinization reduced the content of chl a significantly, with percent reductions of 20%, 25%, and 45%, respectively, at 75, 100, and 120 mM compared to control (Figure 1). On the other hand, chlorophyll b (Chl b) content was not affected by the studied concentration of salinity in comparison with chlorophyll a (Chla) and total carotenoids (TC). In this regard, the degradation of carotenoids was maximized by increasing the level of salt, with percent reductions of 21%, 26%, and 44%, respectively, at levels of 75, 100, and 120 mM, compared to control.

Under salinity stress, the primary metabolites of tomatoes are harmfully affected. Statistical analysis revealed that both soluble carbohydrates (SC) and soluble proteins (SP) were significantly decreased at 100 mM and 120 mM levels. The present reduction was 26% and 44% for sugars as well as 37% and 46% for proteins at 100 and 120 mM, respectively, compared to the control. while a reversible situation was estimated for free amino acid (FAA), where highly significant accumulation was denoted at the levels of 74, 100, and 120 mM, recording an increasing percent of 61%, 66%, and 74% (Figure 2).

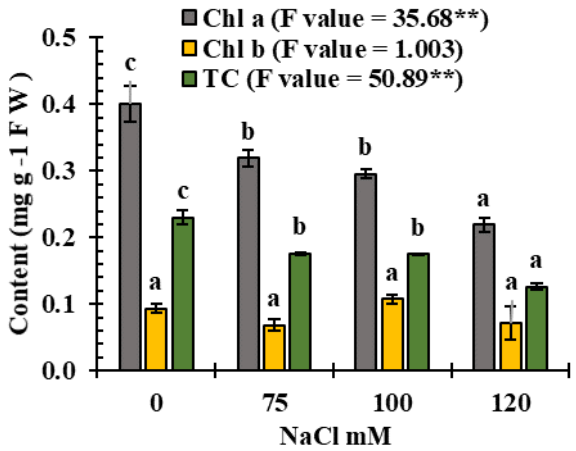

Figure 1. Content of chlorophyll a (Chl a), chlorophyll b (Chl b), total carotenoids (TC) (mg g^{-1} FW). Values are means± SE, n= 3. Mean values with different letters are significantly different at $P \leq 0.05$ according to Tukey's test. * and ** = Significant difference at $P \leq 0.05$ and $P \leq 0.01$ confidence level, respectively.

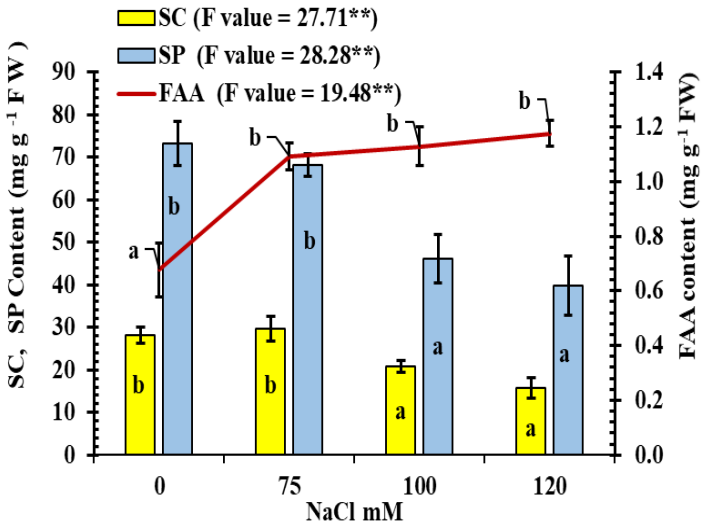

Figure 2. Content of soluble carbohyrates (SC), solube proteins (SP), free amino acids (FAA) (mg g^{-1} FW). Mean values with different letters are significantly different at $P \leq 0.05$ according to Tukey's test. * and ** = Significant difference at $P \leq 0.05$ and $P \leq 0.01$ confidence level, respectively.

The impacts of four different concentrations of salinity (0, 75, 100, and 120 mM) on the characteristics of tomatoes' leaves and their functional groups were investigated using FTIR analysis (Fig. 3) (Table 2). The FTIR spectra showed that no new peaks appeared at 75 and 100 mM compared to the control. While at 120 mM, peaks at 1320 cm^{-1}, and 1380 cm^{-1} were recorded for O–H bending, referring to phenols. The strong peak at 3400 cm^{-1} belongs to O–H and N–H stretching vibrations, referring to alcohol and aliphatic primary amines. The peaks at 2920 cm^{-1} are recorded for C–H stretching, indicating alkanes. Also, the peaks of the C=C functional group were recorded at 1630 cm^{-1} referring to alkene compounds. The peaks at 1430 cm^{-1} have been identified as O–H bending, which pertains to the carboxylic group. The tertiary alcohols and amines, as C–O and C–N functional groups, respectively, were assigned at 1150 cm^{-1}. At 1080 cm^{-1}, the primary alcohol was recorded as the C–O stretching functional group. In addition, the S=O stretching functional group as sulfoxide was noticed at 1030 cm^{-1}.

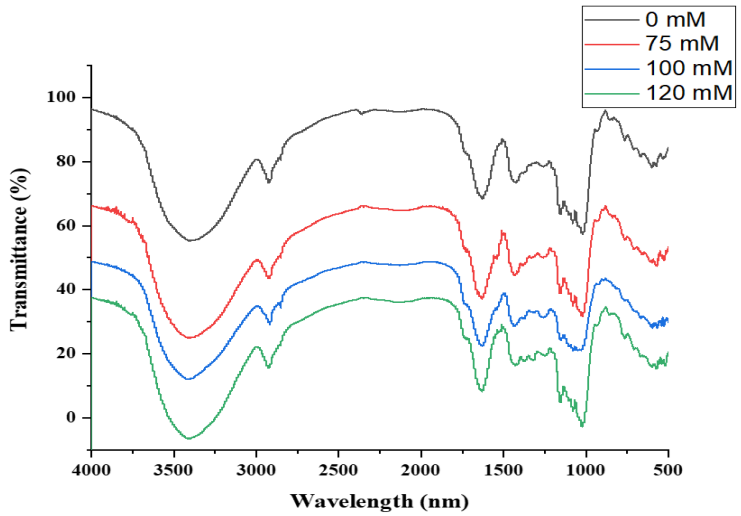

Figure 3. FTIR spectrum of tomatoes' leaves under four levels of salinity (NaCl): 0, 75, 100, and 120 mM.

The data represented in Figure 4 revealed that there is a significant difference in the relative gene expression of polyphenol oxidase (PPO), peroxidase (POD), and thaumatin-like proteins (TLPs) in tomatoes under three different levels of salinity. The relative gene expression of PPO was significantly increased (3.61-fold) at the level of 100 mM compared to the control, without significant effect at the levels of 75 or 120 mM. It was denoted as a reduction in relative gene expression of POD at levels of 75 or 120 mM and an increase of 0.71-fold at 100 mM relative to control. Regarding the relative gene expression of TUB, a non-significant reduction was observed under salinity stress, especially at the lowest saline level applied. However, the relative TLps gene expression was highly significant at levels 75 and 100 mM, about 50.34-fold relative to 120 mM and the control.

Table 3. The functional groups of tomato leaves under four levels of salinity (NaCl 0, 75, 100, and 120 mM) were investigated using FTIR analysis.

Functional groups	Class	Salinity levels mM			
		0	75	100	120
O–H stretching, N–H stretching	Alcohol, Aliphatic primary amines	3400	3420	3420	3430
C–H stretching	Alkanes	2940	2930	2930	2940
C=C stretching	Alkenes	1640	1650	1630	1630
O–H bending	Carboxylic group	1420	1440	1430	1440
O–H bending	Phenol	-	-	-	1380
O–H bending	Phenol	-	-	-	1320
C–O stretching, C–N stretching	Tertiary alcohol, Amines	1150	1160	1160	1160
C–O stretching	Primary alcohol	1070	1080	1080	1070
S=O stretching	Sulfoxide	1030	1030	1020	1030

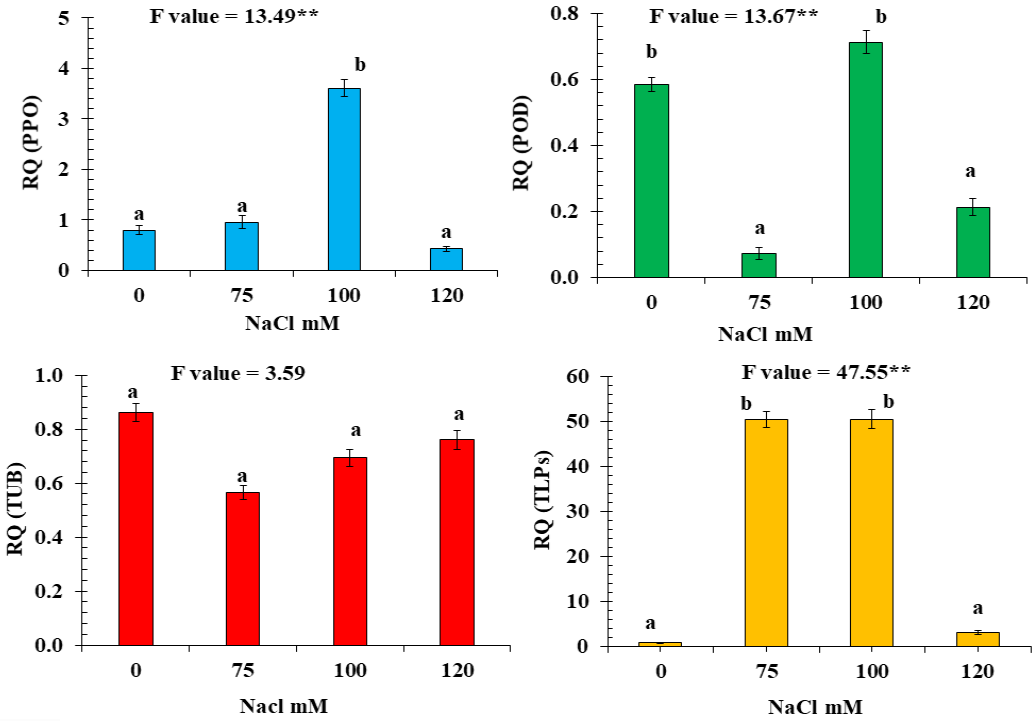

Figure 4. Relative quantitative expression analysis (RQ) of four genes: **A**: polyphenol oxidase (PPO), **B:** peroxidase (POD), **C:** tubulin (TUB), and **D:** thaumatin (TLPs) in tomato plants exposed to salinity at four levels: 0 mM, 75 mM, 100 mM, and 120 mM. Mean values with different letters are significantly different at $P \leq 0.05$, according to Tukey's test. * and ** = significant differences at $P \leq 0.05$ and $P \leq 0.01$ confidence levels, respectively.

Discussion

The saline environment is a persistent stress in arid and semi-arid areas such as Egypt, which is extremely stressful to all life features of sensitive crops such as tomatoes. In the present investigation, salt stress reduced the performance of the shoots and roots of tomato plants, and the reduction rate was intensified by increasing salinity from 100 to 120 mM, while little effect was recorded at 75 mM. In addition, the roots were highly affected by salt stress compared to shoots where the roots were in direct contact with a salt medium. This result is corroborated by observations made during an investigation into the effect of salt stress on avocado root growth, which indicated that root growth in avocados may be more hampered by salinity than shoot growth (Bernstein et al., 2004). Therefore, stress-induced inhibition of root growth and root proliferation could significantly reduce the surface area of juvenile roots and, consequently, the root system's absorption capacity (Wolstenholme & Whiley, 1999). Similar intensification of the damaging impact of salinity on plant growth was reported by Yurtseven *et al.*, who demonstrated that the biomass yield was already diminished at a salinity level of 2.5 dS m^{-1} and that the reduction increased as the salinity rose from 2.5 to 10.0 dS m^{-1}. The average decrease

in biomass yield induced by a salinity increase from 2.5 to 5.0 dS m^{-1} was approximately 37%; as the salinity increased to 10.0 dS m^{-1}, the yield decreased by approximately 60% (Yurtseven et al., 2005). This reduction in tomato biomass could be related to the stress that salt causes on the metabolic activities of cells, which diminishes water potential due to ionic and osmotic stress (Sofy et al., 2022). As most crop plants are sensitive to salinity, which affects plant growth, development, and productivity, improving salt tolerance is crucial for sustaining global agricultural productivity (Raza et al., 2022). Biomass was determined for the reason that this parameter and yield are crucial variables utilized for informed decision-making and production management in agriculture. In addition, biomass measurements offer information on a plant's ability to absorb sunlight, water, and minerals and convert them into plant material, as well as aid in the determination of crop fertilizer and irrigation needs (Johansen et al., 2019). Salt stress usually decreases photosynthesis, but these effects vary with dose and species (Zhang et al., 2018). Salinity stress differentially affected the pigment composition of tomato plants, where Chl b showed high resistance to the applied salt stress, recording no reduction in its content, while Chl a degraded highly significantly even at the lower level of salinity. It was shown that salinity can inhibit chlorophyll production (Khan, 2003), trigger chlorophyllase (Sultana et al., 1999), and cause protein-pigment combinations to be unstable (Jaleel et al., 2008). Also, osmotic stress induced by salinity reduced the water potential and decreased stomatal conductivity, which may limit the flow of CO2 to the leaves, affecting the CO_2 : O_2 ratio in the chloroplasts and hence lowering photosynthesis (Sarker & Oba, 2019). Furthermore, carotenoids were also drastically affected by salt stress, but the damaging impacts showed a moderate response compared to that of Chl a. During salt stress, β-carotene degrades and zeaxanthin synthesis is diminished, resulting in decreased carotenoid levels (Sultana et al., 1999). Thus, tomato plants showed sensitivity to salinity stress, especially at the levels of 100 mM and 120 mM.

The production of suitable osmolytes is one way that plants react to salt stress. Through osmotic adjustment, cells are protected from the oxidative damage induced by reactive oxygen species (ROS) in reaction to high salinity (Abdallah, Abdelgawad, & El-Bassiouny, 2016). The salinity-tolerant ability of plants is controlled by many physiological processes; among them, photoassimilate is inhibited under salt stress, and the degree of reduction in photoassimilate is positively proportional to stress strength (Azzam et al., 2021). This finding is concurrent with the finding of the present study, where soluble sugars have been reduced in response to moderate and high saline treatments. In this respect, researchers found that salt stress reduces photosynthetic pigment and total carbohydrates, which is attributed to the low efficiency of photosystem II (Abdallah et al., 2016). Also, proteins were found to be deregulated by salinity stress at moderate and higher salinities. This reveals the osmotic stress encountered by plants experiencing 100 and 120 mM NaCl salinity. This drift has been reported previously by other researchers; for instance, compared to control seeds at 2, 4, and 6 days, it was found that NaCl-treated seeds had a 16.13%, 26.78%, and 29.49% drop in soluble protein concentration, respectively (L. Chen et al., 2020). In addition, at 12 ds m^{-1} salt stress, soluble protein concentrations were significantly reduced by 38% due to salinity stress (Khan et al., 2022). In contrast, amino acids have accumulated at salinity levels (75, 100, and 120 mM NaCl salinity). In this regard, the accumulation of amino acids could be at the expense of proteins. As the biosynthesis of proteins switches to the accumulation of free amino acids, the faster rate of protein degradation is accompanied by a high amino acid production affinity. It has been reported that amino acids are involved directly or indirectly in controlling plant responses to environmental signals linked to abiotic salinity in terms of osmotic adjustment or scavenging of reactive species (Ragaey et al., 2022). The total content of free amino acids in plants treated with salinity was shown to increase by a statistically significant amount (Selem, 2019). Thus, the level of 75 mM showed little damage from salinity, with no osmotic stress symptoms paralleling the low reduction of tomato performance under stress. These alterations in the contents of the primary metabolites under salt treatments may be associated with the improvement or retardation of the synthesis, accumulation, or expenditure of these cellular metabolites to face the deteriorations induced by salinity stress.

In this study, FTIR analysis revealed that phenolic compounds were generated in tomatoes' leaves to cope with salt stress at 120 mM in comparison to 0, 75, and 100 mM. According to another study, it was shown that salinity stress had an effect on total phenolic chemicals. It was discovered that as plants were irrigated with salt water, their amount increased (Bistgani et al., 2019). It is hypothesized that normal saline tolerance pathway induction occurs in response to moderate salinity stress by elevating total phenolic compound levels (Salem, Msaada, Dhifi, Limam, & Marzouk, 2014). The total phenolic content in red pepper rose under moderate salinity stress, as reported by Navarro et al. In reality, genetics and the environment interact to determine phenolic levels (Navarro, Flores, Garrido, & Martinez, 2006). For protecting against biotic and abiotic stressors, phenolic chemicals are crucial. These metabolites reduce oxidative stress and remove reactive oxygen species (ROS) from various plant tissues, serving as antioxidants (Selmar & Kleinwächter, 2013).

Salinity stress causes secondary stresses on the plants, where activation of oxidative damage is encountered. To protect plant cells from oxidative damage caused by abiotic stresses, such as salt stress, many genes encoding antioxidant enzymes are regulated or deregulated based on the degree of salt stress. Therefore, analyses of the transcriptional levels of antioxidant defense genes could take into account their payoff in the salt stress response in plants. Plant peroxidases have been used as biochemical markers for various types of biotic and abiotic stresses due to their role in very important physiological processes, such as the control of growth by lignification, the cross-linking of pectins and structural proteins in the cell wall, and the catabolism of auxins. In this regard, the damaging impact of high salinity showed a reduction in the level of the relative gene expression of POD, revealing high oxidative damaging impacts at this level compared to control or the level of 100 mM, which kept a higher level of the relative gene expression of POD compared to control but was not significant. On the other hand, the salinity level at 75 mM showed a reduction of relative gene expression of POD compared to the control, revealing low oxidative damage encountered at this level, and maybe other antioxidant genes were stimulated. Regarding the relative gene expression of PPO, the data of the present work revealed that the level of 100 mM showed tolerance to salinity stress, and a high increase in the relative gene expression of PPO was connected to some improvement in POD gene expression. This reveals that the level of 100 mM NaCl adapted to salt stress via increasing PPO and POD relative gene expressions compared to the level of 120 mM, where the oxidative damage reached a threshold that could not be controlled by the cells. In this regard, Azzam *et al.* reported that changes in peroxidase and polyphenol oxidase have a positive correlation with high levels of abiotic stress tolerance (Azzam et al., 2021). It was demonstrated that the expression of POD and PPO genes increased in faba bean cultivars with increasing NaCl concentrations but decreased at high salt concentrations (El-Flaah et al., 2021).

Numerous TLPs have potent antifungal activity and have been engaged in defense mechanisms against a wide range of biotic stressors (de Jesús-Pires et al., 2020). In the present study, we were challenged to study the relative expression of TLPs under salinity stress in tomato plants. Interestingly, the mild and moderate salinity levels folded the relative expression of TLPs compared to the control; however, the expression of the TLP gene was significantly decreased at severe salinity levels compared to 75 and 100 mM salinity levels. This reveals the importance of TLPs in conferring salt tolerance at mild and moderate salinity levels. Some recent studies showed that TLPs were also involved in plant responses to abiotic stress. For example, ectopic expression of a TLP gene in peanuts enhances the tolerance of tobacco seedlings to salt and oxidative stress (Liu et al., 2023). It was demonstrated that turning off TLP6 in rose leaves renders the plants less tolerant to salt stress. Thus, the present studies recommend the importance of TLPs as a salinity tolerance-induced gene at mild and moderate levels (Su et al., 2021).

Osmotic stress is caused by salinity stress, which reduces water availability and hence inhibits plant growth. Salt stress causes significant mechanical stress on plant cells during this phase by raising the pressure threshold for cell walls in growing cells in root and stem meristematic tissues (Neumann, 2011). This mechanical stress modifies cytoskeletal proteins such as tubulin, actin, and kinesins, which assist plants in adjusting to salt stress (Kosová et al., 2013). In the present study, the relative expression of the tubulin gene was included to characterize its role in the salinity tolerance of tomato plants. It is worth mentioning that the tubulin relative expression gene is reduced under soil salinization, especially at a mild level, but such effects are non-significant. It was stated that the importance of tubulin and kinesin in regulating microtubule (MT) organization and ionic homeostasis increases the survival of rice plants under salt stress, thus providing novel genes for salt-insensitive rice breeding in areas with high soil salinity (Chen et al., 2022). The failure of tomato plants to activate the relative expression of the tubulin gene revealed its sensitivity to the applied salinity levels and the damaging impacts of salt on the tomato cells.

Conclusion

To detect the tolerance or sensitivity of tomatoes to salinity, enzyme activity, such as biochemical and gene expression, as molecular markers of peroxidase and polyphenol oxidase, could be used. However, some enzyme activation is responsible for protecting plants against oxidative damage. In addition, the PPO, POD, and TLPs play a vital role in the adaptation of tomatoes against salinity stress at the 100 mM level. In contrast, the expression of the TUB gene in tomatoes does not appear to play a significant role under the studied level of salinity. Such investigations have revealed the important relationship between antioxidant activity and osmolytes, which may be commensurate with its ability to withstand salt stress or because of salt stress, which may cause some change in gene expression.

Recommendations

The research found that severe salinity harmfully affected tomato hybrid T-186. Therefore, it is recommended to transplant tomatoes hybrid T-186 in soils that have salinity not exceeding mild or moderate levels.

Scientific Ethics Declaration

The authors declare that the scientific ethical and legal responsibility of this article published in EPHELS journal belongs to the authors.

Acknowledgments or Notes

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References

Aazami, M. A., Rasouli, F., & Ebrahimzadeh, A. (2021). Oxidative damage, antioxidant mechanism and gene expression in tomato responding to salinity stress under in vitro conditions and application of iron and zinc oxide nanoparticles on callus induction and plant regeneration. *BMC Plant Biology, 21*(1), 597.

Abdallah, M. M. S., Abdelgawad, Z. A., & El-Bassiouny, H. M. S. (2016). Alleviation of the adverse effects of salinity stress using trehalose in two rice varieties. *South African Journal of Botany, 103*, 275-282.

Abdelkhalek, A., Ismail, I. A., Dessoky, E. S., El-Hallous, E. I., & Hafez, E. (2019). A tomato kinesin-like protein is associated with Tobacco mosaic virus infection. *Biotechnology & Biotechnological Equipment, 33*(1), 1424-1433.

Azzam, C. R., Al-Taweel, S. K., Abdel-Aziz, R. M., Rabea, K. M., Abou-Sreea, A. I. B., Rady, M. M., & Ali, E. F. (2021). Salinity effects on gene expression, morphological, and physio-biochemical responses of stevia rebaudiana bertoni in vitro. *Plants, 10*(4), 820.

Bernstein, N., Meiri, A., & Zilberstaine, M. (2004). Root growth of avocado is more sensitive to salinity than shoot growth. *Journal of the American Society for Horticultural Science, 129*(2), 188-192.

Bistgani, Z. E., Hashemi, M., DaCosta, M., Craker, L., Maggi, F., & Morshedloo, M. R. (2019). Effect of salinity stress on the physiological characteristics, phenolic compounds and antioxidant activity of Thymus vulgaris L. and Thymus daenensis Celak. *Industrial Crops and Products, 135*, 311-320.

Chen, G., Xuan, W., Zhao, P., Yao, X., Peng, C., Tian, Y., Chi, W. (2022). OsTUB1 confers salt insensitivity by interacting with Kinesin13A to stabilize microtubules and ion transporters in rice. *New Phytologist, 235*(5), 1836-1852.

Chen, L., Liu, L., Lu, B., Ma, T., Jiang, D., Li, J., Bai, Z. (2020). Exogenous melatonin promotes seed germination and osmotic regulation under salt stress in cotton (Gossypium hirsutum L.). *PloS One, 15*(1), e0228241.

Chomczynski, P., & Sacchi, N. (2006). The single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction: twenty-something years on. *Nature Protocols, 1*(2), 581-585.

Chun, H. J., Baek, D., Jin, B. J., Cho, H. M., Park, M. S., Lee, S. H., Kim, S. T. (2021). Microtubule dynamics plays a vital role in plant adaptation and tolerance to salt stress. *International Journal of Molecular Sciences, 22*(11), 5957.

Dawood, M. F. A., Sofy, M. R., Mohamed, H. I., Sofy, A. R., & Abdel-kader, H. A. A. (2022). Hydrogen sulfide modulates salinity stress in common bean plants by maintaining osmolytes and regulating nitric oxide levels and antioxidant enzyme expression. *Journal of Soil Science and Plant Nutrition, 22*(3), 3708-3726.

de Jesús-Pires, C., Ferreira-Neto, J. R., Pacifico Bezerra-Neto, J., Kido, E. A., de Oliveira Silva, R. L., Pandolfi, V., Pio-Ribeiro, G. (2020). Plant thaumatin-like proteins: function, evolution and biotechnological applications. *Current Protein and Peptide Science, 21*(1), 36-51.

El-Flaah, R. F., El-Said, R. A. R., Nassar, M. A., Hassan, M., & Abdelaal, K. A. A. (2021). Effect of rhizobium, nano silica and ascorbic acid on morpho-physiological characters and gene expression of POX and PPO in faba bean (Vicia faba L.) Under salinity stress conditions. *Fresenius Environ. Bull, 30*, 5751-5764.

El-Khalifa, Z. S., Ayoub, A., & Zahran, H. F. (2022). Estimating the efficiency of summer tomatoes production: A case study in Borg El-Arab Area, Egypt. *Open Journal of Applied Sciences, 12*(6), 855-864.

Fales, F. (1951). The assimilation and degradation of carbohydrates by yeast cells. *Journal of Biological Chemistry, 193*(1), 113-124.

Hafez, E. E., Abdelkhalek, A. A., El-Wahab, A. S. E.-D. A., & Galal, F. H. (2013). Altered gene expression: Induction/suppression in leek elicited by Iris Yellow Spot Virus infection (IYSV) Egyptian isolate. *Biotechnology & Biotechnological Equipment, 27*(5), 4061-4068.

Hsu, J. H., & Lo, S. L. (1999). Chemical and spectroscopic analysis of organic matter transformations during composting of pig manure. *Environmental Pollution, 104*(2), 189-196.

Jaleel, C. A., Sankar, B., Sridharan, R., & Panneerselvam, R. (2008). Soil salinity alters growth, chlorophyll content, and secondary metabolite accumulation in Catharanthus roseus. *Turkish Journal of Biology, 32*(2), 79-83.

Janke, C., & Magiera, M. M. (2020). The tubulin code and its role in controlling microtubule properties and functions. *Nature Reviews Molecular Cell Biology, 21*(6), 307-326.

Johansen, K., Morton, M., Malbeteau, Y., Aragon Solorio, B. J. L., Almashharawi, S., Ziliani, M., Mousa, M. (2019). Predicting biomass and yield at harvest of salt-stressed tomato plants using UAV imagery. *The International Archives of the Photogrammetry, Remote Sensing and Spatial Information Sciences, XLII-2/W13*, 407–411.

Khan, I., Muhammad, A., Chattha, M. U., Skalicky, M., Bilal Chattha, M., Ahsin Ayub, M., Rahman, M. A. (2022). Mitigation of salinity-induced oxidative damage, growth, and yield reduction in fine rice by sugarcane press mud application. *Frontiers in Plant Science, 13*, 865.

Khan, N. A. (2003). NaCl-Inhibited Chlorophyll Synthesis and Associated Changes in Ethylene Evolution and Antioxidative Enzyme Activities in Wheat. *Biologia Plantarum, 47*(3), 437-440.

Kosová, K., Prášil, I. T., & Vítámvás, P. (2013). Protein contribution to plant salinity response and tolerance acquisition. *International Journal of Molecular Sciences, 14*(4), 6757-6789.

Lee, Y. P., & Takahashi, T. (1966). An improved colorimetric determination of amino acids with the use of ninhydrin. *Analytical Biochemistry, 14*(1), 71-77.

Lichtenthaler, H. K. (1987). [34] Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes. In *Methods in Enzymology* (Vol. 148, pp. 350-382): Academic Press.

Liu, Q., Sui, X., Wang, Y., Zhu, M., Zhou, Y., & Gao, F. (2023). Genome-Wide Analyses of Thaumatin-like Protein Family Genes Reveal the Involvement in the Response to Low-Temperature Stress in Ammopiptanthus nanus. *International Journal of Molecular Sciences, 24*(3), 2209.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. *Methods, 25*(4), 402-408.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry, 193*, 265-275.

Navarro, J. M., Flores, P., Garrido, C., & Martinez, V. (2006). Changes in the contents of antioxidant compounds in pepper fruits at different ripening stages, as affected by salinity. *Food Chemistry, 96*(1), 66-73.

Neumann, P. M. (2011). Recent advances in understanding the regulation of whole-plant growth inhibition by salinity, drought and colloid stress. *Advances in Botanical Research, 57*, 33-48.

Parvin, K., Hasanuzzaman, M., Bhuyan, M. B., Mohsin, S. M., & Fujita, M. (2019). Quercetin mediated salt tolerance in tomato through the enhancement of plant antioxidant defense and glyoxalase systems. *Plants, 8*(8), 247.

Ragaey, M. M., Sadak, M. S., Dawood, M. F. A., Mousa, N. H. S., Hanafy, R. S., & Latef, A. A. H. A. (2022). Role of signaling molecules sodium nitroprusside and arginine in alleviating salt-Induced oxidative stress in wheat. *Plants, 11*(14), 1786.

Raza, A., Tabassum, J., Fakhar, A. Z., Sharif, R., Chen, H., Zhang, C., Varshney, R. K. (2022). Smart reprograming of plants against salinity stress using modern biotechnological tools. *Critical Reviews in Biotechnology*, 1-28.

Salem, N., Msaada, K., Dhifi, W., Limam, F., & Marzouk, B. (2014). Effect of salinity on plant growth and biological activities of Carthamus tinctorius L. extracts at two flowering stages. *Acta Physiologiae Plantarum, 36*(2), 433-445.

Sarker, U., & Oba, S. (2019). Salinity stress enhances color parameters, bioactive leaf pigments, vitamins, polyphenols, flavonoids and antioxidant activity in selected Amaranthus leafy vegetables. *Journal of the Science of Food and Agriculture, 99*(5), 2275-2284.

Schlegel, H. G. (1956). Die Verwertung organischer Säuren durch Chlorella im Licht. *Planta, 47*(5), 510-526.

Selem, E. (2019). Physiological effects of Spirulina platensis in salt stressed Vicia faba L. plants. *Egyptian Journal of Botany, 59*(1), 185-194.

Selmar, D., & Kleinwächter, M. (2013). Influencing the product quality by deliberately applying drought stress during the cultivation of medicinal plants. *Industrial Crops and Products, 42*, 558-566.

Sheteiwy, M., Ulhassan, Z., Qi, W., Lu, H., AbdElgawad, H., Minkina, T., Josko, I. (2022). Association of jasmonic acid priming with multiple defense mechanisms in wheat plants under high salt stress. *Frontiers in Plant Science*, 2614.

Sofy, M., Mohamed, H., Dawood, M., Abu-Elsaoud, A., & Soliman, M. (2022). Integrated usage of Trichoderma harzianum and biochar to ameliorate salt stress on spinach plants. *Archives of Agronomy and Soil Science, 68*(14), 2005-2026.

Su, L., Zhao, X., Geng, L., Fu, L., Lu, Y., Liu, Q., & Jiang, X. (2021). Analysis of the thaumatin-like genes of Rosa chinensis and functional analysis of the role of RcTLP6 in salt stress tolerance. *Planta, 254*(6),

Sultana, N., Ikeda, T., & Itoh, R. (1999). Effect of NaCl salinity on photosynthesis and dry matter accumulation in developing rice grains. *Environmental and Experimental Botany, 42*(3), 211-220.

Wolstenholme, B. N., & Whiley, A. W. (1999). Ecophysiology of the avocado (Persea americana Mill.) tree as a basis for pre-harvest management. *Revista Chapingo Serie Horticultura, 5*, 77-88.

Yurtseven, E., Kesmez, G. D., & Unlukara, A. (2005). The effects of water salinity and potassium levels on yield, fruit quality and water consumption of a native central anatolian tomato species (Lycopersicon esculantum). *Agricultural Water Management, 78*(1), 128-135.

Zhang, Y., Kaiser, E., Zhang, Y., Yang, Q., & Li, T. (2018). Short-term salt stress strongly affects dynamic photosynthesis, but not steady-state photosynthesis, in tomato (Solanum lycopersicum). *Environmental and Experimental Botany, 149*, 109-119.

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