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Neurobehavioral Toxicity of 4-methylimidazole on NMRI mice: Using Behavioral and Histopathology Methods

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Abstract: 4-Methylimidazole (4-MEI) a toxic compound in humans and animal is formed as by product of the Maillard reaction. It is found in many foods and beverages and as an intermediate in the synthesis of different materials. Current study was conducted for evaluation the toxicity effects of 4-MEI (70,150,200 mg/kg) on behavioral changes using open field (OF), elevated plus maze (EPM), passive avoidance, Y-maze test and in the following, abnormalities in dentate gyrus cell of the hippocampus tissue using haematoxylin-eosin (H&E) staining. Our results showed that 4-MEI increased the total distance movement and decreased the time spent in central zone in the OF test while in the EPM test, in a dose-dependent manner decreased the time spent in the open arms. Also, 4-MEI led to different in size and density of dentate gyrus cell which was related with disruption obtained in passive avoidance test. Findings obtained indicated 4-MEI can induces neurobehavioral toxicity by increase in locomotor activity, anxiety and memory disorder in mice. Therefore it can be mentioned 4-MEI may have neurotoxicity and results of this study will be useful in order to investigation regarding probable mechanisms involved in neurobehavioral disorders in rats exposed 4-MEI.

Keywords: 4-Methylimidazole, Neurotoxicity, Behavioral test, H&E staining

Introduction

4-Methylimidazole (4-MEI) has been identified as undesirable by-products of Millard reaction by chemical reactions between carbohydrates, especially reducing sugars, and ammonia or amino acids in various food products such as caramel coloring, soy sauce and wine (Jacobson, 2012). 4-MEI has wide application in various industries and as raw material use in the pharmaceutical, photographic, dyeing and agricultural chemicals (Chan 2004) (Sivertsen et al. 2009, Mehri et al. 2020b). Because of the toxicity of 4-MEI on health human, a couple of studies have investigated toxicity of this compound on various tissues (Mehri et al. 2020b) Beerves et al. (2016) showed 4-MEI can inhibit functional of the cytochrome P450 - in the human liver tissue (Narwal et al., 2001). In similar study, different researchers were reported that 4-MEI may cause adenoma and carcinoma in the mouse respiratory system (Chan et al., 2008) mononuclear cell leukemia (Mehri et al. 2020b) hepatotoxicity and inflammation in male and female rats (Chan et al., 2008). In goats and heifers, intravenous administration of 4-MEI can lead to salivation, defecation, coughing, urination, and convulsions (Nielsen et al., 1993). According to previous studies, 4-MEI can penetrate the brain tissue and cause developmental toxicity and cytotoxicity in animals (Mehri et al. 2015, Mehri et al. 2020b). Our previous study has shown that 4-MEI could be delivered to brain cells and affect the electron transfer chain in isolated mitochondria which resulted in oxidative stress events (Mehri et al. 2015). Also 4-MEI causes restlessness, frothing at the mouth, bellowing, paralysis, excitation, and convulsions in rodents. These effects are a result of the inhibition of GABA synthesis in the brain of rodents (Sivertsen et al., 2009). Based on published studies, there were no research regarding neurobehavioral toxicity of 4-MEI in human. There were various techniques for neurobehavioral assessment in animals. As mentioned in previous studies, in order to investigation behavioral changes including disorder in

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short or long term memory, anxiety, movement disorders, and spontaneous alternation behavior have been used of Passive avoidance, elevated plus maze, open field and Y-maze test ,respectively(Narwal et al., 2001, Hidaka et al., 2011). From one side, due to the increasing demand for consumption of caramel colors as the most important source of 4-MEI in across the world and on the other, because of the possible various toxic effects of 4-MEI on human health, 4-MEI has received much attention and requires additional research (Mehri et al. 2020a). Also to date, according to my knowledge there was no about neurobehavioral toxicity of 4-MEI in NMRI mice, therefore, in the present study, for the first time, neurobehavioral toxicity of 4-MEI on NMRI mice was conducted using open field, elevated plus maze, passive avoidance and Y-maze test and, in the following, abnormalities in dentate gyrus cell of the hippocampus tissue was assessed using haematoxylin-eosin (H&E) staining in different mice.

Method

Materials

4-MEI, scopolamine and midazolam were purchased from (Sigma-Aldrich, USA). Prepared solutions of 4-MEI (70, 150 and 200 mg/kg) were given to form oral once a day for - 14 days to mice and -then all experimental were done in the 15 day of treatment. (1mg/kg) of midazolam were administered to form intraperitoneal 30 min before - the passive avoidance test as positive control for assess long term memory (LTM) behavior in animals (Savić et al., 2005)and also (1mg/kg) of scopolamine were given as negative control in y-maze test for assays spontaneous alternation behavior. The control group only received vehicle for the duration 14 days.

Animal treatment schedule

In current study, NMRI mice with different weighing (20 to 25g) obtained from Razi institute, Iran was used for perform experiments. The various groups of animals were placed in cage (n=10) with controlled temperature (22±2 °C), relative humidity (40–50%), with a standard diet of food and water. After a one-week handling period, the experiment was done on the mice. All the experimental were accomplished in accordance with to the ethical standards approved by -the Board of Animal Research of Shahid Beheshti University of Medical Sciences, Iran. All the ethical concerns were cautiously adhered. In order to determination of optimum dose, a pilot study of dosing was done. For this goal, animal were randomly placed in two groups of control and test containing six animals in each. The best dose for 4-MEI obtained 150 mg/kg that is equal to1/3 of its LD50 (357 mg/kg) that was according to previous study(Sivertsen, Nygaard et al. 2009). 4-MEI dissolved in the corn oil and was given orally through gavage for 2 weeks to treatment groups while the control group received equal amount of the vehicle.

Study design

Mice in each group (I-IV, n=10) received the treatment as described below:

Group I (control group): received single dose of corn oil, (as 4-MEI solvent) through oral gavage for 2 weeks once a day.

Group II (DZN -intoxicated): received single dose of 4-MEI (200 mg/kg body weight/-day through oral gavage) once daily for 2 weeks once a day

Group III (DZN -intoxicated): received single dose of 4-MEI (150 mg/kg body weight/-day through oral gavage) once daily for 2 weeks once a day

Group IV (DZN -intoxicated): received single dose of 4-MEI (50 mg/kg body weight/-day through oral gavage) once daily for 2 weeks once a.

At the end of the 14 day of treatment, all behavioral tests was done then, animals anesthesia by ketamine, sections from the brain was separated and fixed in formalin the remaining tissue stored at -20°C for histopathological study.

Behavioral study

Locomotor activity test

The open-field (OF) test was conducted to assess the locomotor activity of animals to investigate a novel environment. The OF test was done in cages - to form (40×40×40cm) as described by Onishchenko et al., (2011). After administering 4MI for a duration of 14 days, in day 15 test, the NMRI mice were transferred to a sterilized cage and the movement of each animal was documented for 10min using a camera that was positioned on top of the cages. In this test, parameters including the time spent in the peripheral zone, the total distance moved and the central zone for each mouse were estimated and reported using the software EthoVision (Noldus, Netherlands) and the results were applied for statistical tests (Onishchenko et al., 2011).

Y-maze test

This experiment was used to evaluate the spontaneous alternation behavior of animals. The maze of this device was made of plastic with three horizontal arms radiating from a central platform. Animals move freely through one arm of the maze for 10 min, and then the number of arm entries was noted by the experimenter. Accepted alternation is documented as multiple entries into the three arms (A, B and C), after each experiment, the arms were decontaminated with 20% ethanol to eliminate any body odors of the animal. The findings were stated using the mentioned equation (Hidaka, Suemaru et al. 2011):

$$\text{Percentage of alternation} = \left[\frac{\text{number of accepted alternations}}{\text{whole arm entries} - 2} \right] \times 100.$$

Elevated plus maze test

The experiment of (EPM) was conducted to evaluate unconditioned anxiety-like behavior in an animal according to the method presented by (Kuleshkaya et al., 2011) (Kuleshkaya et al., 2011). EPM was made of open arms (n=2), enclosed arms (n=2), and also a central platform. The arrangement of the four arms in the maze was in a manner that the arms were placed opposite to each other. - At this test, the animal was placed in the center of the maze - and the movement of animals was recorded for 5 min. The possible anxiety of mice treated with 4MI was assessed. Anxious mice abstain from open arms and desire to remain more duration in closed arms, and then the duration of the time spent in the open and closed arms by mice was estimated using The EthoVision (Version 8, Netherlands).

Passive avoidance test

This test was used to assess long term memory (LTM) in animals (Vollala et al., 2010). The device was made from a bright and dark section that were disconnected by a partition with a door of guillotine material. The dark section was fortified with an electric network line. The test was done during two days. In the first day, we placed the animal in the light section and were allowed to explore the environment for 30s. Subsequently, we opened the door and the animal was allowed to move freely in the dark compartment. When each mouse entered the shock compartment an electrical shock (0.5mA, 2s) was given to the mice. In the second day, the mice were placed in the light section and then the door was opened after 30s. Latency time to enter the dark compartment was documented. The cut-off time in this examination was 300s (n=10) (Narwal et al. 2012; Trněčková et al., 2005).

Haematoxylin-eosin staining

After behavioral testing, animals (the ones that received vehicle and 4MI 200mg/kg/day) were separated and the brain tissue of animals was excised. Subsequently, fixation of brain tissue was done using a 4% paraformaldehyde solution for 48 h and placed in paraffin. 10µm sections of paraffin blocks were prepared by a microtome device (Leica Germany). Sections obtained of the hippocampus were stained using haematoxylin-eosin, based on the method previously explained by Drury and Wallington (Drury and Wallington). The evaluation of the dentate gyrus cell was done by light microscopy with magnification (×40) (24).

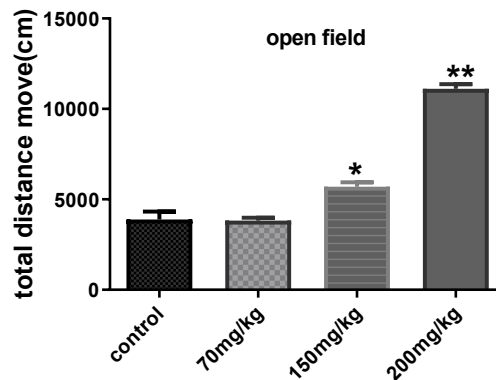
Results and Discussion

Statistical analysis

OF test

The open-field test was performed to evaluate activity and behavioral response of animals to a novel environment. According to Fig.1A, 14-day treatments with 4-MEI in various concentrations significantly changed the total distance moved and the time spent in the center than the control group. OF test showed significant increase in the total distance moved in mice that received 200 ($p<0.05$) and 150 ($p<0.01$) mg/kg than the control group. In addition, the mice that received high doses of 4-MEI spent less time in the central zone than the control group ($p<0.05$ and $p<0.01$ respectively, Fig. 1B).

A)



B)

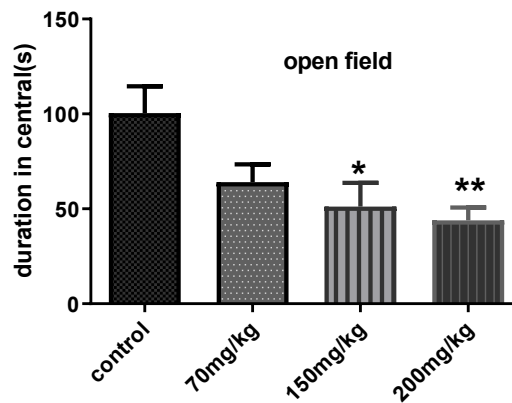


Fig. 1. Effects of 4-MEI on total distance moved (A) and duration in central (B) in OF test. Results are indicated as mean \pm SEM of 10 separate animals in each group. * $p<0.05$, ** $p<0.01$.

Y-maze test

In y-maze test, there was not significant different in spontaneous alternation between animals in the control group and the animals exposed with 4-MEI at the different doses ($p>0.05$, Fig. 2).

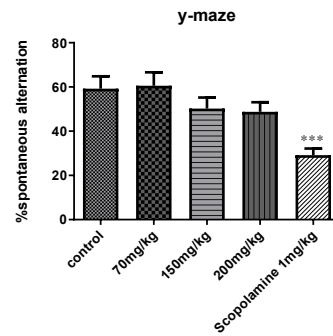
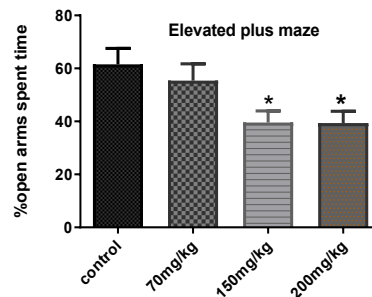


Fig. 2. Effects of 4-MEI on the percentage of spontaneous alternation in Y- maze test. Data are presented as mean \pm SEM of 10 separate animals in each group. *** $p < 0.001$ shows significant change than the control group.

Elevated plus maze

Elevated plus maze was performed to assess unconditioned anxiety. The 14- day treatments significantly changed the percentage of time spent in the open arms. 4-MEI at doses 150 and 200 mg/kg/ significantly reduced the percentage of time spent in open arms ($p < 0.05$ for both, Fig 3A) and increased the number of closed arms entries ($p < 0.01$ for both, Fig. 3B) than the control.

A)



B)

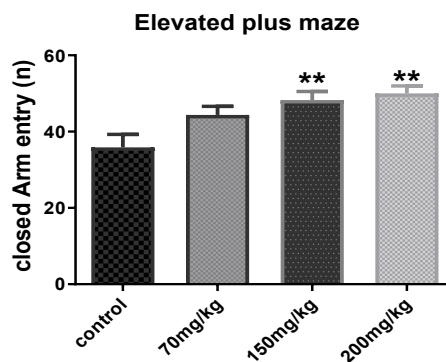


Fig. 3. Effects of 4-MEI on the %time spent in open arms (A) and number of the closed arms entries in EPM test (B). Results are indicated as mean \pm SEM of 10 separate animals in each group. * $p < 0.05$, ** $p < 0.01$.

Passive avoidance

Passive avoidance test is believed to evaluate the long term memory in mice. In this test, significant change was identified between the various groups using the ANOVA test in the avoidance latency. The mice treated with different doses of 4-MEI for fourteen days indicated a significant reduction in avoidance latency time than the control group Fig. 4 ($p < 0.001$ for all).

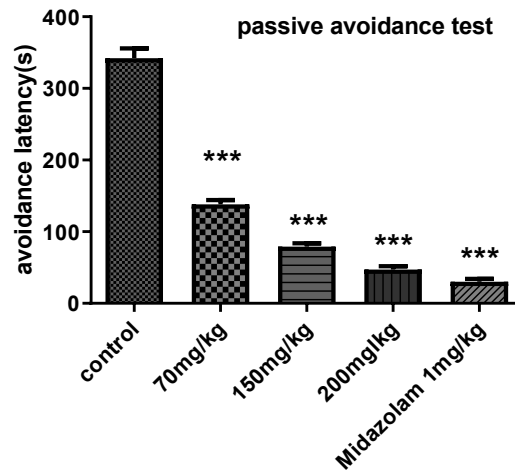


Fig. 4. Effects of 4-MEI on the avoidance latency time in passive avoidance test. Midazolam was used as a standard drug. Reports are expressed as mean \pm SEM of 10 separate animals in each group *** $p < 0.001$.

Histology

Fig. 5A and B show staining of the dentate gyrus cells of the hippocampus area of the control group and 4-MEI exposed mice (200mg/kg/day) using haematoxylin-eosin respectively. Significant reduction in nuclei size and of dentate gyrus cells was observed in the mice treated with 4-MI compared to the control mice.

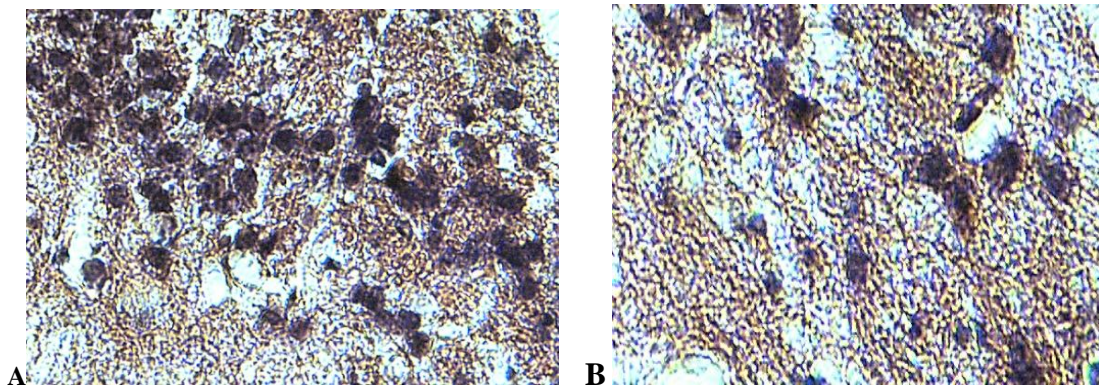


Fig. 5. Effects of 4-MEI on nuclei size reduction and condensation of dentate gyrus cells in the hippocampus of the control $n=10$ (A) and 4MI $n=10$ (200 mg/kg/day) (B) exposed mice. The tip of the arrow in the shapes represents the dentate gyrus cells ($\times 40$).

Discussion

Recently, 4-MEI has raised great concern among federal and state regulatory agencies because of its toxicity, carcinogenicity and presence in foods and beverages (Bu et al. 2015). According to published studies, the original toxicity source of 4-MEI is ammoniated forage and caramel coloring (Sivertsen et al. 2009). Owing to its high potential of carcinogenicity and toxicity in humans, 4-MEI were been considered by the National

Cancer Institute for various study (Chan, 2004). In this study we evaluated the neurobehavioral toxicity of 4-MEI using various behavioral tests, our results showed that 4-MEI induces neurobehavioral disorders by increase in locomotor activity, anxiety, extinction memory, and spontaneous alternation in mice. This results obtained was agreement with our previous reports that indicated 4-MEI can induce toxicity in brain tissue by mitochondrial dysfunctions and neurobehavioral toxicity in pregnant mice (Mehri et al. 2015). According to results of OF test, dietary exposure to various doses of 4-MEI for 14 days increased locomotion activity due to increase level of anxiety in the animal. In line with our study, Chan 2008 showed that 4-MEI induced annexations and high activity after oral exposure in rat. The results obtained of this search confirmed our findings (Chan et al. 2008).

The results of the EPM test for assess the level of anxiety showed that 4-MEI -treated mice compared to the control group in a dose-dependent manner tend to spend less time in open arms and trend for stay in closed arms .These results may confirm the knowledge that 4-MEI can lead to anxiety in mice and produce neurologic effects. According to the detected results in the passive avoidance test regarding long term memory, 4-MEI could disrupt in long term memory function in treated mice compared to the control group. This results was confirmed with the reports of previous studies that have demonstrated the capability of 4-MEI in inducing neurologic effects in goats and rabbits (Nielsen et al. 1993).Consistent with these results, the data obtained of H&E staining showed that 4-MEI could cause some disturbance in dentate gyrus cells. The dentate gyrus (DG) cell is an important section in hippocampal structure (Kempermann, 2002), and has key role in learning and memory processing (Mehri et al. 2020a,b). Thus, the disorder produced in dentate gyrus (DG) cell involved in learning zone could be an important reason for long-term memory impairment in passive avoidance test.

As seen in this study and also mentioned form previous reports, symptoms of clinical neurotoxicity in animals exposed to 4-MEI including confusion, tremors, increase in locomotor activity and anxiety in mice may be consistent with the depletion of cerebral GABA levels induced by the inhibitors of the cerebral glutamic acid decarboxylase (GAD) activity and development of excitation in animals (Sivertsen et al., 2009). Several studies showed a link between Ca² influx and GABA receptor-mediated neurodegeneration (Weiss & Sensi, 2000, Shimohama, 2009). Inhibition of the GAD activity might result in marked intracellular Ca² accumulation and mitochondrial ROS production. The other proposed mechanism of 4-MEI is oxidative injury induced in mitochondria of brain tissue is related with our previous study (Mehri et al. 2020a). In this study, was indicated that the 4-MEI can change motor activity, learning activities and anxiety in a dose-dependent manner. Finally, since this compound is produced as a by-product and has many applications in production of caramel coloring, soy, ammoniated molasses, dyes, and rubber and can produce many hazards on veterinary and human health, therefore, more future studies are needed to identify the possible role of 4-MEI in neurotoxicity using in vivo and in vitro methods, which may provide insight into the role of 4-MEI in neurodegenerative effects in humans.

Conclusion

Our results indicated that the exposure to 4-MEI significantly increases the total distance movement and decreases the time spent in the central zone in the OF test. Also 4-MEI increases the time spent in the open arms in treated group than the control group in the EPM test. It be mentioned that, 4-MEI induced reduction in nuclei size and increase in condensation of the dentate gyrus cells of hippocampus which is associated with disruption in long-term memory in passive avoidance test. Therefore high doses of 4-MEI can disrupt neurobehavioral functions by degenerating hippocampus neural cells.

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Conflict interest

All authors express that they have no potential conflict of interest.

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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