EXPERIMENTAL STUDY

Delineating the antigenotoxic and anticytotoxic potentials of 4-methylimidazole against ethyl methanesulfonate toxicity in bone marrow cell of swiss albino mice

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ABSTRACT

4-Methylimidazole (4-MEI) is mostly used in beverages and coloring food, dark beers and common brands of cola drinks, which may contain more than 100 μg of this compound per 12-ounce serving. This study was aimed to investigate the antigenotoxic and anticytotoxic effects of 4-MEI (100, 130 and 160 mg/kg) against ethyl methanesulfonate (240 mg/kg) using chromosome aberrations (CAS) and Mitotic index (MI) tests in bone marrow cells of Swiss Albino Mice at 12 h and 24 h treatment periods. So, the t-test was used for the statistical analysis. In this research, 4-MEI at all concentrations for 12 h treatment period reduced chromosomal aberrations and at 130 and 160 mg/kg concentrations for 24 h treatment period increased chromosomal aberrations induced by EMS (240 mg/kg), but these reductions and increases were not significant. Also, intraperitoneal injection of 4-MEI at doses of 100, 130 and 160 mg/kg combined with EMS (240 mg/kg) showed that the mitotic index was decreased at 100 and 130 mg/kg for 12h and 130 mg/kg for 24 h treatment periods, when compared to positive sample (EMS), but did not show any statistically different from the EMS treated group. It can be concluded that 4-MEI might not be antigenotoxic and protective effects in bone marrow cells of Swiss Albino Mice, because 4-MEI could not reduce the chromosomal aberrations induced by EMS. This investigation was supported by a grant from Cukurova University science research project (FDK-2014-2617).

Introduction

Food coloring is used both in commercial food production and in household cooking (1). More than 2,500 items of food coloring additives are used for various purposes, including coloring and increase nutrient value (2). One of food color is 4-Methylimidazole (4-MEI), which has a yellow color. 4-MEI was utilized by IARC (International Agency for Research on Cancer) in 2011 and set into group 2B2 (3). 4-MEI is used as a chemical intermediate, crude material or component in the manufacture of pharmaceuticals, photographic and photothermographic chemicals, dyes and pigments and agricultural chemicals (4). 4-MEI is unintentionally found in our foods. Caramel color (which is the most used beverage coloring and food), dark beers and common brands of cola drinks may comprise more than 100 μg of this compound per 12-ounce serving (5). Ishie and al reported that LD50 values of 4-MEI are 370 mg/kg orally and 165 mg/kg intraperitoneally for mice; 120 mg/kg intraperitoneally for rabbits; and 590 mg/kg orally and 210 mg/kg intraperitoneally for chickens (6). NTP (The National Toxicology Program) reported a two-year consuming cancer assessment of 4-MEI in mice and rats. The results of this research showed a clear evidence of carcinogenic activity of 4-MEI in male and female B6C3F1 mice based on increased incidences of alveolar/bronchial neoplasms. In accordance with this result, the NTP found an equivocal evidence of carcinogenic activity in female rats (F344/N) based on increased incidences of mononuclear cell leukemia and no evidence of carcinogenic activity in male rats. However, the manufacture of certain artificial caramel colorings can lead to the formation of carcinogens (4, 7). In contrast, other experimental studies reported that 4-MEI provide chemopreventive effects against some cancer (7, 8). Decreased incidences of tumors in rats were mentioned in the NTP results, but they were not given much prominence, because the NTP commonly focuses on cytotoxic identification rather than cancer prevention (4, 7).

CAS (chromosome aberrations) is a widespread method for study of many drugs and other material on genetic material or chromosome (9). Antigenotoxic effect of some food coloring were reported from other researchers. For example, chemopreventive activity of chlorophyllin was showed by Drosophila system (10, 11, 12). Similar results were observed by Izawa et al (1997), who observed red and yellow pigments from Monascus, which has an inhibitory effect

KEY WORDS: 4-Methylimidazole, ethyl methanesulfonate, antigenotoxicity, anticytotoxicity, chromosome aberration.
against the bacterial mutagenicity of heterocyclic amines (13). In the view of the extensively use of 4-MEI by humans, it would be of interest if further studies were carried out for obtaining more information on the possible in vivo antigenotoxic effects of 4-MEI against other genotoxic materials. Consequently, this study was aimed to investigate the antigenotoxic effects of 4-MEI against ethyl methanesulfonate (EMS) using chromosome aberrations (CASs) and Mitotic index (MI) tests in bone marrow cells of Swiss Albino Mice.

Materials and methods

Chemicals

In this research, the test substance (4-MEI) was purchased from Sigma and its properties are shown in Figure 1.

Lot No: 08302BF
Chemical Formula: C4H6N2
Molecular Weight: 82.11
Synonym: 1H-Imidazole, 4-methyl (9CI); imidazole, 4-methyl; 4(5)-methylglyoxaline; 4(5),4(5)-methylimidazole; 5-methylimidazole
Trade name: 4-MEI
PubChem: 13195
Molecular weight: 82.10 g mol⁻¹
Appearance: Slightly yellowish solid
Density: 1.02 g/cm³
Melting point: 46 to 48 °C (115 to 118 °F; 319 to 321 K)
Boiling point: 263 °C (505 °F; 536 K)
CAS Number: 822-36-6
Purity: 98 %

In this study, colchicine (CAS No. C-9754), NaCl (CAS No. 7647-14-5), KCl (CAS No. 7447-40-7) ethyl methanesulfonate (EMS) (CAS No. 62-50-0) were purchased from Sigma and Gibmsa (CAS No. 1092040100) was purchased from Merck and all test solutions were freshly prepared prior to experiment.

Experimental animals

Male and female Swiss Albino Mice, 6–8 weeks old and weighing about 33–40 g, were obtained from the Experimental Research and Application Centre of Çukurova University, Turkey. They were kept in polypropylene shoebox cages with a grill top and were aclimatized to the control diet for 1 week. Animals were fed with the standard diet and water. Three animals were housed per cage and were maintained in a controlled environmental condition of temperature and (22 ± 2 °C) humidity (45–60 %) on alternatively 12 h dark/light cycles. The study was apprised by the Çukurova university Institutional animal Ethics Committee (FDK-2014-2617) and all experiments were accomplished in accordance with the advisors of the ethics committee.

EMS administration

Ethyl methanesulfonate (EMS) well-known mutagenic and clastogenic agents in in vivo test system were used as a genotoxic agent (14). Sub-lethal high doses of EMS (240 mg/kg b.w) was employed (12 or 24 hours before sacriﬁed of the animals), as earlier established by Riaz and Vasudev (15). The required volumes of EMS (0.5 mL) were dissolved in double distilled water administered intraperitoneally (i.p.).

Experimental protocol

After the acclimatization period (one week), animals were treated through intraperitoneal injections with 100, 130 and 160 mg/kg body weight were administered. 4-MEI administered as single dose mixed with 240 mg/kg EMS in 0.5 mL per mouse intraperitoneal to 6 animals (three male and three female). The experimental animals were divided into nine groups, each group comprising of six animals (three male and three female)

1. Control group (untreated control): The control groups only received normal diet (pellet and water).
2. Positive control group (12 hours treatment): The positive control groups received a single intraperitoneal injection of 240 mg/kg Ethyl methanesulfonate dissolved in double distilled water 12 hours before test (12 hours before sacriﬁed).
3. Group 3: Received a mixture of 100 mg/kg 4-MEI and 240 mg/kg EMS dissolved in double distilled water 12 hours before test (12 hours before sacriﬁed).
4. Group 4: Received a mixture of 130 mg/kg 4-MEI and 240 mg/kg EMS dissolved in double distilled water 12 hours before test (12 hours before sacriﬁed).
5. Group 5: Received a mixture of 160 mg/kg 4-MEI and 240 mg/kg EMS dissolved in double distilled water 12 hours before test (12 hours before sacriﬁed).
6. Positive control group (24 hours treatment): The positive control groups received a single intraperitoneal injection of 240 mg/kg Ethyl methanesulfonate dissolved in double distilled water 24 hours before test (24 hours before sacriﬁed).
7. Group 7: The a mixture of 100 mg/kg 4-MEI and 240 mg/kg EMS dissolved in double distilled water 24 hours before test (24 hours before sacriﬁed).
8. Group 8: Received a mixture of 130 mg/kg 4-MEI and 240 mg/kg EMS dissolved in double distilled water 24 hours before test (24 hours before sacriﬁed).
9. Group 9: Received a mixture of 160 mg/kg 4-MEI and 240 mg/kg EMS dissolved in double distilled water 24 hours before test (24 hours before sacriﬁed).

In this research, colchicine (3 mg/kg body weight: 0.5 mL), a spindle fiber inhibitor that arrests cells at metaphase was also administered by intraperitoneal 2 hours before the animals were sacriﬁed (16). So, animals were sacriﬁed by cervical dislocations at 12 hours and 24 hours (four groups 12 hours and four groups 24 hours) after the treatments and bone marrow cells were harvested.
Preparation of bone marrow metaphase chromosome

These animals were sacrificed by cervical dislocation and bone marrow chromosomal aberrations assay was employed to determine any clastogenic effects in dividing cells, which were in the metaphase. The bone marrow was processed and slides were prepared by routine standard air dry technique (17). In this method, the femur bones were dissected from the animals and the bone marrow was aspirated using physiologic serum (5 ml). The suspension was then centrifuged for 5 minutes at 2000 rpm then, the supernatant was decanted. After that, the cells treated with 0.4 % KCl (37 °C, for 35 minute) as the hypotonic solution and the suspension was centrifuged for 10 minutes at 1200 rpm and supernatant was decanted. The cells fixed in cold methanol: glacial acetic acid (3:1) for 20 min at room temperature. The treatment with fixative was repeated three times. Then the cells were spread on cold glass slides (4 °C) and air-dried. The slides were stained with Giemsa (5 % in Sorensen buffer) for 8 min (18).

Chromosomal aberration analysis

One hundred metaphases of the bone marrow cells were analyzed for the presence of CA (chromosomal aberrations). The CA was classified according to the ISCN (International System for Human Cytogenetic Nomenclature) (19). The number of the CA was obtained by calculating the percentage of metaphases at each concentration and treatment period that showed structural chromosome aberrations. Structural CAs scoring was done in the following categories: chromatid-type aberrations (exchanges, breaks and sister union) and chromosome-type aberrations (rings, breaks and dicentrics). Chromosome aberrations were evaluated in 100-well-spread metaphases per animal (in total, 600 metaphases per concentration or treatment periods) (20). Gaps were not evaluated as CA, according to Mace et al (21).

Mitotic index analysis

Mitotic index (MI) was analyzed in order to understand the effect of 4-MEI, EMS mixture on cell proliferation in different treatment and different times. Mitotic index was determined by analyzing 3000 cells from each animal and scoring the cells that were in metaphase. The Mitotic index was calculated by using the formula below (22).

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\text{Mitotic Index} = 100\times\frac{\text{cells in metaphase}}{3000}
\]

Statistical analysis

Student t-test was utilized for establishing the statistical significance of chromosome aberration and mitotic index data obtained from microscopic analyses were compared to the corresponding control and the positive control groups. Concentration–response relationships were determined from the correlation and regression coefficients for the percentage of cells with CA, as well as for the mean MI (23, 24).

Results

The results of chromosome aberrations analysis in male and female Swiss Albino Mice bone marrow cells at metaphase stage when intraperitoneally injection only EMS as a dose (240 mg/kg body weight) and 4-MEI at various doses (100, 130 and 160 mg/kg body weight) combined with EMS (240 mg/kg body weight) are summarized in Table 1.

The result of this study showed that the EMS significantly induced the CAs at 12 and 24h treatment periods in positive control sample. In the combination groups (4-MEI + EMS) except 160 mg/kg for 12h and 100 mg/ for 24 h percentage of CAs was significantly increased when compared to the control. 4-MEI + EMS as a mixture induced structural CAs as the positive control at 160mg/kg in the 24 h treatment period. In addition, this increment in the CAs was found to be concentration-dependent for 24h treatment period (p < 0.252) (Fig. 2). 4-MEI decreased the genotoxicity of EMS at all concentrations for 12h treatment period in the bone marrow cells of Swiss Albino Mice, but this decrease was not significant when compared to the positive control (EMS). This means that 4-MEI might not have inhibition of chromosomal damage induced by EMS.

The effects of EMS as a dose (240 mg/kg body weight) and 4-MEI at various doses (100, 130 and 160 mg/kg body weight) combined with EMS 240 mg/kg body weight by intraperitoneally injection on the mitotic index are shown in Table 2. The mitotic index (MI) of EMS and EMS plus 4-MEI treated groups was decreased and showed statistically significant differences from the untreated control. In the combination groups, the MI was decreased at100 and 130 mg/kg for 12 h and 130 mg/kg for 24 h treatment

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**Table 1. CA in bone marrow cells of Swiss Albino Mice treated with 4-MEI + EMS.**

| Treatment | Test substance | Time (hr) | 4-MEI+EMS Conc. (mg/kg) | Chromatid type | Chromosome type | with aberrations ±SE |
|-----------|----------------|-----------|------------------------|----------------|----------------|-----------------------|
| Control   | -              | -         | 0                      | 6              | 1.00±0.258     |
| EMS       | 12             | 24        | 20                     | 3              | 3.83±0.401a,   |
| 4-MEI+EMS | 12             | 24+240    | 12                     | 5              | 2.83±0.307a,   |
| 4-MEI+EMS | 12             | 130+240   | 15                     | 7              | 3.66±0.422a,   |
| 4-MEI+EMS | 12             | 160+240   | 16                     | 0              | 2.67±0.558     |
| EMS       | 24             | 240+240   | 16                     | 2              | 3.00±0.447a,   |
| 4-MEI+EMS | 24             | 100+240   | 15                     | 3              | 3.00±0.577     |
| 4-MEI+EMS | 24             | 130+240   | 19                     | 0              | 3.16±0.543a,   |
| 4-MEI+EMS | 24             | 160+240   | 22                     | 3              | 4.16±0.792a,   |

Data are expressed as the mean values (±SE) obtained from six mice bone marrow cells; n = 6, a: significant from negative control; b: significant from positive control (EMS), a,b; p < 0.05; a,b; p < 0.01; a,b; p < 0.001.
periods when compared to the positive control groups (240 mg/kg EMS), but did not show any statistically significant difference from the EMS treated group. In addition, no concentration-dependent effect was observed in the obtained results.

**Discussion**

The relationships between food, nutrition and cancer, and the knowledge that cancer might be a preventable disease has resulted in an increased interest in studying the mutagenic or antimutagenic potential of some dietary constituents (25). Considerable emphasis has been laid down on the use of dietary constituents to prevent the mutagen induced mutation or chromosomal damage due to their relative nontoxic effects. Ethyl methanesulfonate is well-known mutagenic and clastogenic agent in mice bone marrow test system. EMS was often used as a positive control in genotoxic test, both in vitro and in vivo test system. The types of chromosomal aberrations induced by EMS as a positive control were reported to be chromosome break, chromatid break (14). According to our knowledge, this is the first study that addresses the antigenotoxic effects of 4-MEI in bone marrow cells of Swiss Albino Mice. In the present study, the results showed that intraperitoneally injection of 4-MEI at doses of 100, 130 and 160 mg/kg combined with EMS (240 mg/kg) for 12 h treatment period was found to reduce chromosomal aberrations induced by the EMS, but these reductions were not significant. According to the findings of this research, it can be said that 4-MEI did not have antigenotoxic effect in bone marrow cells of Swiss Albino Mice. Many color additives including anthocyanin, annatto and bixin appear to be potent antimutagens and antigenotoxics against genotoxic reagents (26, 27, 28). In contrast to 4-MEI, some of the food coloring have antigenotoxic effect against other genotoxic reagent. For example, Izawa et al (1997) observed that that red and yellow pigments from *Monascus* have an inhibitory effect against the bacterial mutagenicity of heterocyclic amines. Similarly, Edenharder and Tang (1997), reported the antimutagenic effect of purpurin, alizarin and other 10 anthraquinone compounds using the Amastest (29). In another study, chemopreventive activity of chlorophyll was shown by *Drosophila* system (10, 11, 12). The number of reviews concerning the carcinogenicity and mutagenicity 4-MEI has been published. For example, The National Toxicology Program (NTP) in the study of the rodent cancer bioassay for 4-MEI in male and female animals (B6C3F1 mice). The results of this research showed that the carcinogenic activity of 4-MEI in male and female mice and led to the development of the lung tumors (8). Similar results were obtained by Hannah et al (2010) since both Coke and Pepsi soft drinks, which contain the same substance (4-MEI) induced the chromosomal abnormalities in the roots of *Allium cepa* treated for 2, 24, and 48 hours treatment times (30). Another study, Jensen et al (1983) reported that caramel was the important color additive in Cola soft drinks and it showed a mutagenic activity in *Salmonella typhimurium* TA 100 (31). On the other hand, results obtained by Rayes (2008), showed that the Cola soft drink had toxic effects on the mice testicular cells (32). Overall, these available studies provided little evidence for the genotoxicity of 4-MEI.

The mitotic index is simply a measurement to determine the percentage of cells undergoing mitosis. Mitosis is the division of somatic cells when genetic information from one single cell is equally dispersed into two daughter cells. The mitotic index may be elevated during necessary processes to life, such as the normal growth of plants or animals, as well as cellular repair the sire of an injury (33). Cytotoxic effect is measured by mitotic index and other tests. In our research, we also used this parameter. Intraperitoneally injection of 4-MEI at doses of 100, 130 and 160 mg/kg combined with EMS (240 mg/kg) showed that the MI was decreased except 160 mg/kg 4-MEI + EMS (240 mg/kg) for 12 h and 100 and 160 mg/kg 4-MEI + EMS (240 mg/kg) for 24 h treatment periods when compared to the positive control (240 mg/kg EMS), but did not show any statistically significant differences from the EMS treated group. The neurotoxic syndrome of 4-MEI observed by Hidaka (1976) occurred soon after a high gavage dose of 4-MEI under conditions, where both metabolism and renal clearance were saturated (34). Imidazole’s, especially those substituted at the 4-position, have been recognized as inhibitors of cytochromes P450. Hargreaves et al (1994) reported that 4-MEI was a strong inhibitor of p-nitrophenol hydrolase in rat liver. p-Nitrophenol is a cytochrome P450 2E1 substrate (35). 4-MEI forms complexes with heme-containing enzymes such as cytochrome P450 and results in an inhibition of mixed function oxidase activity (36). Supporting the present study, probable cytotoxic effect of 4-MEI has also a similar mechanism.

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**Fig. 2.** The percentage of abnormal cells in Swiss Albino Mice treated with 4-MEI+EMS for 24 h treatment period (p < 0.180).

**Tab. 2.** MI in bone marrow cells of Swiss Albino Mice treated with 4-MEI+EMS for 12 and 24 h.

| Test substance | Time (hr) | 4-MEI+EMS Conc. (mg/kg) | MI±SE |
|---------------|-----------|------------------------|-------|
| Untreated Control | -         | -                      | 5.532±0.315 |
| EMS           | 12        | 240                    | 2.217±0.294 a |
| 4-MEI+EMS     | 12        | 100+240                | 2.683±0.273 a |
| 4-MEI+EMS     | 12        | 130+240                | 1.943±0.230 a |
| 4-MEI+EMS     | 12        | 160+240                | 1.915±0.263 a |
| EMS           | 24        | 240+240                | 2.320±0.182 a |
| 4-MEI+EMS     | 24        | 100+240                | 2.308±0.334 a |
| 4-MEI+EMS     | 24        | 130+240                | 2.912±0.439 a |
| 4-MEI+EMS     | 24        | 160+240                | 2.250±0.279 a |

Data are expressed as the mean values (±SE) obtained from six mice bone marrow cells; n = 6, a: significant from negative control; b: significant from positive control (EMS). a, b: p < 0.05; a,b,c: p < 0.01; a,b,c,d: p < 0.001.
Conclusion

In this research, no significant difference in CA and MI values was observed among the animals that received the EMS alone or in combination with 4-MEI (at various doses). It can be concluded that 4-MEI might not have antigenotoxic and protective effects in bone marrow cells of Swiss Albino Mice. However, it must be investigated in other test systems.

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