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EXPERIMENTAL STUDY

Investigation of anticarcinogenic and antioxidant effects of 4-methylimidazole

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ABSTRACT

4-methylimidazole is widely used in pharmaceuticals, photographic and agricultural chemicals. The substance is extensively found in many human and animals foods. In this research, anticancer effect of the 4-MEI was studded using MTT test using MCF-7 cell line. Effect of the 4-MEI on apoptosis or necrosis was analyzed by DNA fragmentation assay using Swiss Albino rats as a model organism. Antioxidant effect of the substance was investigated by assaying protective effect of the substance on circular plasmid DNA against H_2O_2 as an oxidative agent.

4-MEI showed inhibitory effect on proliferation of MCF-7 cell line by all concentrations and the decrement was significant and concentration dependent. Result of DNA fragmentation assay showed 4-MEI concentrations dependent of smear formation showing necrotic effects of the 4-MEI on mouse cells. Also, the 4-MEI showed a good antioxidant activity and protective effect against H_2O_2.

CONCLUSION: The result of this study showed that 4MEI has significant antioxidant and anti-cancer effect. Also, according to the result, 4-MEI has necrotic effects on mouse cells *(Fig. 3, Ref. 21)*. Text in PDF *www.elis.sk.*

KEY WORD: 4-MEI, MCF-7, fragmentation, antioxidant, anticancer.

Introduction

4-methylimidazole, 4-MEI or 4-MeI, is a heterocyclic organic chemical compound and it is formally derived from imidazole through replacement of the hydrogen in position 4 by a methyl group. 4-Methylimidazole is used widely in pharmaceuticals, photographic and agricultural chemicals (NTP, 2007). Also, the substance is extensively found in many human and animals foods. The concentration of the 4-MEI in plasma and milk of the dairy cow fed from ammoniated forage containing 4-MEI is 0.28 and 2.7, respectively (Muller et al, 1998). The 4-MEI is found in natural dyes during the ammonia and ammonia-sulfite caramelization process of carbohydrates. Caramel colors of foods have been grouped in 4 classes by the Joint FAO/WHO Expert Committee on Food Additives (JECFA), and the European Union Scientific Committee for Food, two of them (III and IV) are prepared using compounds that contain ammonia (Houben and Penninks, 1994).

Class III ammonia caramels (containing 4-MEI) are widely used in various bakery products, cola drinks, vinegars, dark-brown beers etc. Dark beers and common brands of cola drinks may contain more than 100 µg of this compound per 12-ounce serving (Jacobson et al, 2011). Their use accounts for 20–25 % of the total use of caramel coloring in the USA and for about 60% in Europe. Class IV ammonia-sulfite caramels (containing 4-MEI) are used in soft drinks, pet foods and soups (Houben and Penninks, 1994), and account for approximately 70 % of the caramel coloring produced worldwide (Licht et al, 1992a). However, some researchers have reported regarding to the detrimental effect of the commonly used substance, for example, Maekawa et al demonstrated that 4-MEI csused tumor formation in F344 rats and Chan et al (2008) published that mononuclear-cell leukemia was significantly higher in rats feeding 4-MEI than the control animals. The carcinogenic effect of the 4-MEI on mice was reported by Hagiwara et al (2003).

Previously, the toxic effect of the 4-MEI on immune system of human had been reported (Aguilar, et al, 2011). Nevertheless according to my knowledge there are not enough studies on the protective effect, such as antioxidant and anticancer effect of the 4MEI, so by considering the extremely usage of the 4-MEI in worldwide, it was decided to investigate the protective effect of the substance. 4-MEI is widely used by people and liver and breast cancers are common in our countries. So it was decided to in vitro analyze the anti-cancer effect of 4-MEI by MTT test using mcf-7 cell line and the effect of the 4-MEI on DNA fragmentation on liver of rats with cancer by treatment with 1, 2 di methyl hydrazine.

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Materials and methods

Materials

In this study, 4-MEI (CAS number: 822-36-6, purity: 98 %, molecular weight: 82.11) was purchased from Sigma Aldrich and used as a test substance. The molecular structure of the substance as shown in Figure 1. In this research pET22b plasmid was purchased from Novagen and MTT dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), dimethylsulfoxide (DMSO), mouse embryo 3T3- L1 cells were purchased from the Sigma and RPMI 1640 medium was purchased from Life technologies.

Analysis of anti-cancer effect of 4-MEI by MTT method

The mouse embryo MCF-7 cells were obtained from Department of Medical Biology, Medicine Faculty of Cukurova University, Adana, Turkey. The culture medium included Dulbecco's RPMI1640 medium. The cell culture condition was 37 °C in humidified 5 % CO_2 incubator. The inhibition of cell proliferation was evaluated by MTT assay. The MTT assay was performed according to the method from Mosmann (1983).

MCF-7 pre adipocytes were plated into microtiter plates at a density of 1×104 cells/well. After 24 h, culture medium was replaced by 90µL RPMI 1640 combined with 300, 450, 600, 750 µg/mL of the 4-MEI (The tests for each concentration was repeated 8 times) and the cells were incubated for 24 and 48 hours (one group for 24 h and the other for 48 h). The above mentioned concentrations were opted due to the LD50 (750 µg/ml) of the substance which was obtained by pre-tests in our lab. After the incubation time, 10µl sterile filtered MTT solution (5 mg/mL) in phosphate buffered saline (PBS, pH = 7.4) was added to each well and the cells were incubated for 5 hours, then unreacted dye was removed. The insoluble formazan crystals were dissolved in 200 µL/well DMSO and measured spectrophotometrically in Medispec Esr-200 spectrophotometer at 570 nm.

The relative cell proliferation (%) was calculated by equation of: A570nm (melanin) / A570nm (untreated control) x 100

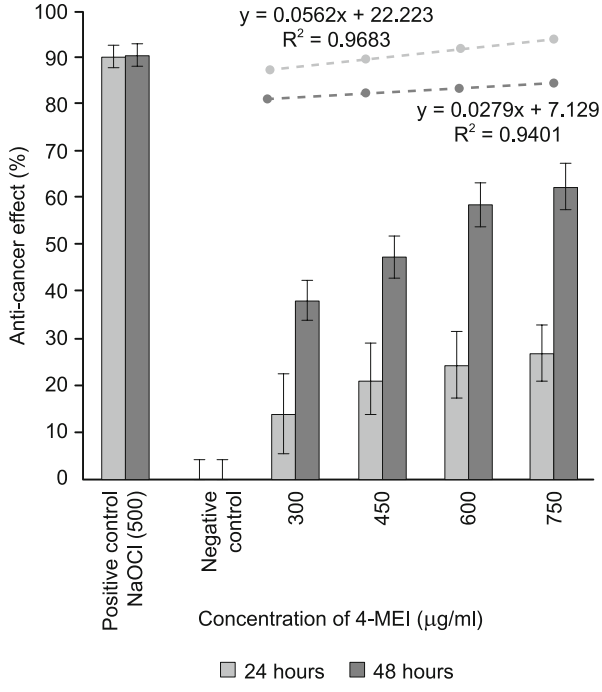

Fig. 1. MTT assay of the 4-MEI on MCF-7 cell line.

Statistical analysis

Statistical analysis was performed using Student's t-test. In this research, statistical tests were performed using Minitab software (Norizadeh Tazehkand and Topaktas, 2014).

Protection of plasmid DNA against oxidative agent

Protective effect of 4-MEI on DNA against oxidative agent was evaluated using pET22b plasmid DNA (Novagen). The experiments were performed in a volume of 10 µl in a microfuge tube containing 3 µl pET22b (150 ng) plasmid DNA, 1 µl of H_2O_2 (30 %), 1 µl of $FeSO_4$ (1 mM), and 7 µl of 4-MEI in the concentrations of 300, 450, 600 and 750 µg/ml, respectively. In this research, untreated controls (untreated pET22b plasmid DNA) and positive controls (1 µl of H_2O_2 (30 %), 1 µl of $FeSO_4$ (1 mM) and 3 µl pET22b plasmid DNA,) were also used. The reactions were incubated at room temperature for 30 min. After incubation, the reaction mixture (5 µl) along with gel loading dye (6×) was loaded on a 1.8 % agarose gel for electrophoresis (Tepe 2011).

Assessment of apoptosis and necrosis by DNA fragmentation assay

In this part of the study, 32 Swiss Albino rats were grouped in the 4 groups of 8 rats (4 males and 4 females).

1st group as a negative control group was been fed with commercial pellet diet and water for 10 weeks. 2nd group as a positive control was peritoneally administered 4-methyl hydrazine (mg/kg 4) two time per week during the first two weeks. After that, the animals were fed with commercial pellet diet and water. After receiving 4-methyl hydrazine (mg/kg 4) two time per week for two weeks, the 3th and 4th groups were intraperitoneally administered 25 mg/ml and 50 mg/ml of 4-MEI, respectively for eight weeks.

After completing of 10 week period, the animals were sacrificed by cervical dislocations and their liver tissue were separated and stocked at –80 °C. In order to DNA fragmentation analysis, the DNA were extracted from the tissue and tested in agarose gel electrophoresis (Negoescu, 1996, Fehsel, 1999)

Result

Study of anti-cancer effect of 4-MEI by MTT test

In this study, treatment of MCF-7 cell line by 4-MEI for 24 h showed a decreased cell proliferation up to 14.21 %, 21.34 %, 24.44 % and 27.13 % by concentration of 300 µg/ml, 450 µg/ml, 600 µg/ml and 750 µg/ml respectively. Also, for 48 hour exhibited 38.18 %, 47.51 %, 58.59 % and 62.56 % by concentrations of 300 µg/ml, 450 µg/ml, 600 µg/ml and 750 µg/ml respectively (Fig. 1). In the control group that was treated with NaCl, the decrement

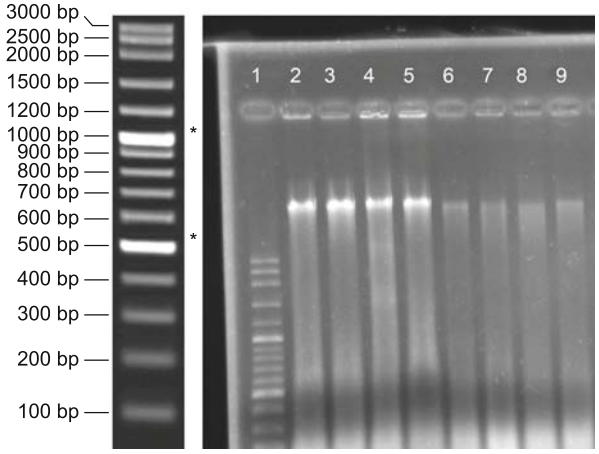

Fig. 2. The effect of 4-MEI on DNA fragmentation. Lane 1, Marker; lane 2 and 3, negative control; lane 4 and 5, positive control; lane 6 and 7, samples treated with 4-MEI (25 mg/kg); lane 8 and 9 samples treated with 4-MEI (50 mg/kg).

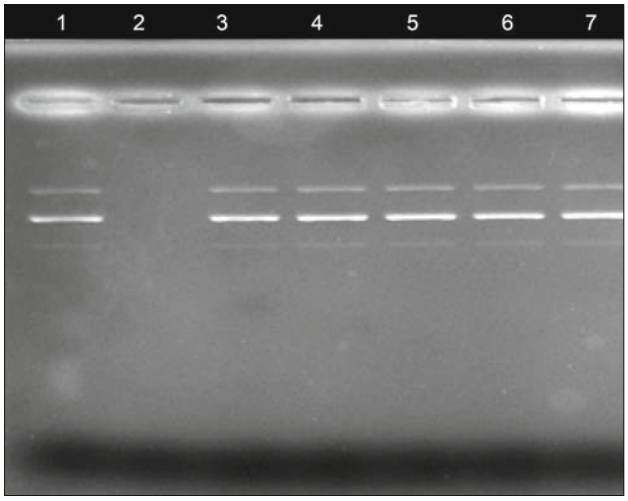

Fig. 3. Electrophoretic pattern of pET22b plasmid DNA after treatment with 4-MEI and H_2O_2. Lane 1: pET22b plasmid DNA (untreated control); lane 2: plasmid DNA treated with H_2O_2 and $FeSO_4$; lanes 3: pET22b plasmid treated with H_2O_2, $FeSO_4$ and 4-MEI (300 µg/ml); lanes 4: pET22b plasmid treated with H_2O_2, $FeSO_4$ and 4-MEI (450 µg/ml); lanes 5: pET22b plasmid treated with H_2O_2, $FeSO_4$ and 4-MEI (600 µg/ml); lanes 6: pET22b plasmid treated with H_2O_2, $FeSO_4$ and 4-MEI (750 µg/ml).

was 94.93 %. Therefore, the 4-MEI showed an inhibitory effect on proliferation of MCF-7 cell line by all concentrations and the decrement was significant and concentration dependent.

Assessment of apoptosis and necrosis by DNA fragmentation assay

Genomic fragments of irregular sizes and a DNA smear in gel electrophoresis are generally related to the DNA fragments originated by necrosis and apoptosis, respectively. Gel electrophoresis analysis showed that 4-MEI at 50mg/kg concentration clearly produced characteristic DNA smear and no DNA laddering (200bp). Also, the formation of DNA smear was dependent on 4-MEI concentrations. According to the results, 4-MEI has necrotic effects on mouse cells (Fig. 2).

Antioxidant effect of the 4-MEI

Figure 3 shows the electrophoretic pattern of plasmid DNA after 4-MEI and H2O2 treatment. pET22b plasmid DNA (untreated control) showed three bands on agarose gel electrophoresis comprised of super coil, open supercoil and linear DNA. The pET22b plasmid treated with H_2O_2 and $FeSO_4$ (positive control) showed the fragmentation and elimination of DNA on the agarose gel indicating that the OH° generated from H_2O_2 broke DNA strand. Electrophoretic pattern of DNA treated with H_2O_2 and $FeSO_4$ along with 4-MEI (concentrations of 300, 450, 600 and 750 µg/ml) showed three bands on agarose gel electrophoresis as untreated control, comprised from super coil, open supercoil and linear DNA (Fig. 3). Therefore, 4-MEI protected the DNA against oxidative agent (OH°).

Discussion

In this research, the 4-MEI caused a decline of cell proliferation in the cancer cell line MCF-7, although it did not led to apoptosis of the cells. Rayes et al. (2008) showed that cola had ant-bacterial effect on *Bifidobacterium bifidum* and *Lactobacillus acidophilus.* Also, it had cytotoxic effect on mouse testis cells. Cola drinks may contain more than 100µg of this compound per 12-ounce serving (Jacobson and Michael, 2011). Murray (2010) in his paper has written from National Toxicology Program (NTP) that 4-MEI had anticancer effect on fibroadenoma. In the paper published by Takemoto (2002) it has been written that the 4-MEI had antibacterial and antifungal effect because it made the bacterial and fungal plasma membrane to be permeable, which caused a cell death. Similarly, liver hypertrophy in mice following intraperitoneal administration of 4-methylimidazole has been reported by Hidaka et al (1976). In hypertrophy, the cell volume is increased because of absorbing more food or water.

In this research, the 4-MEI showed a considerable antioxidant activity similarly, in the research carried out by Kohen et al (1988) 2-MEI, and Imidazol showed 28 % and 39 % antioxidant activity, therefore the antioxidant activity of the 4-MEI is originated from Imidazol. Abdel-Wahab et al (2011) published that imidazole-based compounds showed a considerable antioxidant activity as reflected in the ability to inhibit lipid per-oxidation in rat brain and kidney homogenates and rate erythrocyte hemolysis.

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