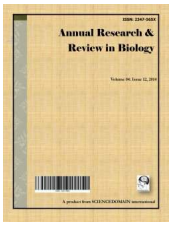

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Effects of Methyl Tert-butyl Ether (MTBE) on the Mucosal Immunity in the Small Intestine of the White Albino Mice

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Authors' contributions

This work was carried out in collaboration between all authors. Author LMMA designed the experiments and performed histological studies. Author ZYAS performed animal treatment experiments. Author HAM analyzed the data. Author HHA wrote and prepared the manuscript's first draft. All authors read and approved the final manuscript.

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ABSTRACT

Some chemical compounds have a significant effect on living organisms. This is shown in the physiological and biological changes that appear on some cells and tissues of the body. The current study aimed to investigate the effect of the dissolved MTBE in drinking water on the mucosal immune cells of the small intestine of the white mice. The presence of dissolved MTBE at a concentration of 5000 ppm in drinking water has significant increase (P <0.02) of mast cells in the mucosa of mice. Similarly, at a concentration of 2500 ppm a significant increase (P <0.01) of goblet cells were observed in treated rats compared with untreated rats. The Experiment has also shown that the effect of MTBE in drinking water included the weights of animals in general, where the weight gain was weak in the treated rats compared to those where the drinking water was free of the compound. Another observation of the effects of MTBE in drinking water was the increase of the

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weights of some internal organs (e.g. liver, kidneys and heart) in the treated animals, as well as effects on the behavior of these animals with an obvious fatigue was observed on treated individuals. The chronic exposure of intestinal tissues to MTBE may led to inflammatory effects and /or tissue damages, and as a response the mucosal innate immunity, particularly the numbers of mast, Paneth and goblet cells were increased in synergistic fashion to play their protective role in lessen the damages caused by MTBE.

Keywords: MTBE; intestinal immunity; mast cells; goblet cells; paneth cells.

1. INTRODUCTION

Methyl tert-butyl ether (MTBE) is a volatile oxygenate that is added to gasoline to enhance total burning of fuel that leads to reduction of the concentration of carbon monoxide and other harmful exhaust emission from vehicles. MTBE is used as substitute for the tetraethyl lead in unleaded gasoline to improve air quality by reducing harmful emissions and ozone precursors and increase the efficiency of fuel oxidation, making it the fourth most important chemical product in the United States since 1970s [1,2]. Despite the use of MTBE in the USA is forbidden since 2003, and its use in many European countries has been considered as controversial due to various reports of its carcinogenicity, a number Asian and Middle Eastern countries (e.g. China and Saudi Arabia) still using MTBE in unleaded gasoline [3].

Alarming levels of MTBE have been recorded in the air of many urban cities in the USA and other European countries. In addition, the presence of high concentrations of MTBE in drinking water supplies (tap, ground and surface water) has been recorded in various cities within the USA, Canada, and European countries such as the Netherland and Germany. In order to protect the people from the adverse carcinogenic and non-carcinogenic effect of MTEB in contaminated drinking water, the USEPA has recommended that MTBE levels in contaminated drinking water should not exceeds 40 µg / L [1,4,5,6,7,8,9].

Various Studies have shown that chronic or sub-chronic exposure to MTBE, ETBE and related compounds by means of either inhalation or drinking MTBE-, ETBE-contaminated water has serious negative impacts on human health [10]. Human health effects of MTBE may include difficulty of breathing, fatigue, dizziness, and nausea these were reported after surveying a number of individuals who had exposure to MTBE by inhalation [10]. Non-carcinogenic and carcinogenic effects expected on humans after chronic and sub-chronic exposure to MTBE and related compounds were supported by studies on animal models (e.g. rats, mice) exposed to MTBE-contaminated drinking water. Sprague-Dawley rats receiving MTBE in drinking water for three months were found to have mitochondrial energy failure [11]. Male rats that were administered MTBE for four weeks by gavage where shown to have reproductive system malfunction due to toxicity induced by MTBE [12]. The development of testicular tumors; mononuclear leukemia and kidney tumors in rats were significantly increased after chronic exposure to MTBE [13].

Mast cells (mastocyte) are white blood cells that are part of the immune system, despite being known for their role in allergy (as it is rich in histamine), they play an important protective role such as wound healing and defense against pathogens. Goblet cells are glandular, columnar epithelial cells that produce mucus to protect mucous membranes such as the inner surface of the intestine, the secretion of this mucus act as lubricate and protect the walls of these membranes, the secretion of mucus by goblet cells is usually stimulated by bacterial and viral pathogens. Paneth cells together with other cells (e.g. goblet cells and enterocytes) represent the main component of the epithelium of the small intestine. Paneth cells contain granules that consist of anti-microbial compounds and play an important role in the immunity and host defense in intestinal area [14,15,16,17,18,19].

Due to the high solubility properties of MTBE in water, contaminated drinking water display the most direct exposure route of this toxic compound via its entry into the gastrointestinal tract through the absorption areas of the small intestine. Since the immune cells in the small intestine (e.g. Mast, goblet and Paneth cells) are the first defense line to confront any foreign microorganism entering the bloodstream through absorption in the bowel area, the current study was aimed to determine the effects of MTBE on

a these immune cells in the small intestine, effects on the overall body weights of the rats and internal organs weights will also be studied.

2. MATERIALS AND METHODS

2.1 Test Animals

Twenty-five female albino mice (strain Balb/C) were purchased from King Fahad Medical Research Centre at the King Abdulaziz University (Jeddah). Mice were allocated into five groups at cages (A – E) each of which contain five individuals under conventional animal house conditions. Food and water were supplied at all times and the bedding was changed twice weekly. Animals were left for two weeks for adaptation with Terramycin treatment (Pfizer, oxytetracycline hydrochloride, 3.0 g L^{-1}) given in drinking water to ensure the animals are free from any microbial infections prior to begin the experiment. They were then returned to normal drinking water for one week prior to start MTBE-treatment in water [20].

2.2 Treatment of Mice with MTBE

Animals in each group where given different dosage of MTBE with their drinking water. Group A was supplied with only drinking water as a control group and groups B to E were given concentration of 1000, 2500, 5000 and 10,000 ppm of the MTBE in the drinking water respectively. Water depletion was completed at the same ratio of the compound in the treated groups for up to one month and then the animals were killed with an overdose of chloroform to obtain tissues and organs.

The experiments were performed in accordance with the guidelines for the care and use of laboratory animals of Umm Al-Qura University, Faculty of Applied Science, Institutional Animal Care Use Committee (IACUC), Saudi Arabia.

2.3 Fixation and Staining of Tissues

At autopsy, two pieces of tissue were taken from the small intestine (each about 1.0 cm^2), approximately first third below the pyloric sphincter. Each one centimeter section of the intestine was destined for different treatment. Two fixatives used were Carnoy's and 10% formalin [21]. Carnoy's-fixed samples were kept from 4-6 hours in the fixative and then transferred to 70% ethanol solution. Formalin-fixed samples were kept in formalin until processing. Tissues then were washed thoroughly with 70% ethanol solution then dehydrated through graded alcohol (70%, 80%; 90%; 95%; and 2X absolute ethanol) followed by three changes of xylene and finally two changes of paraffin polymer at 56ºC. Samples remained in each solution for one hour before passing on to the next treatment. Tissues were embedded in pure paraffin wax, left to dry, then, mounted on small wooden blocks and sectioned at 6 µm using a microtome then mounted on slides for staining [21].

Sections were deparaffinised by heat to 50ºC on a wired hotplate, followed by 5 min in each of two xylene washes followed by two min washes in each of absolute ethanol, 90%; 80%; 70% and 50% then finally dipped into distilled water. Sections of samples fixed in Carnoy's were placed into a coplin jar full of pre-warmed (50ºC) Alcian blue and left for 45 min. The tissue was then counterstained by immersion in another jar full of pre-warmed Safranin O for 5-10 min. The slides were then dehydrated by a rapid dip through different grades of ethanol starting from distilled water and through to absolute ethanol for not more than 1.5 min each. Sections were transferred through fresh xylene three times for clearing, and then mounted using DPX mounting solution. Other formalin fixed samples were divided into two groups. First were dipped into Alcian blue (pH 2.5) for 5-10 min then washed in distilled water to remove excess stain and treated with 1.0% periodic acid for 5-10 min. Sections were then washed for a second time in tap water, then placed in Schiffs reagent for 5-10 min. Next they were washed in tap water until they appeared pink-colored. Finally, sections were dehydrated through different grades of ethanol, as described above, cleared in fresh xylene and mounted for cell analysis. Sections were dewaxed in xylene then hydrated through graded alcohol to water, stained in Carazzi's haematoxylin for 10 min, then moved straight into 1.0% HCl in 70% alcohol for a few seconds. Next, they were washed in tap water, moved to phloxin stain and left for 20 min, rinsed in tap water, then blotted dry and moved to Tartrazine solution for not more than 10 min under microscopical observation. Sections were then rinsed in 95% alcohol and then twice in absolute ethanol. Finally, the sections were cleared and mounted in DPX. The method used to count the cells was based on a Weibel 2 graticule [22], it was calibrated as described in Alkazmi [21].

2.4 Determination of Changes in Mast Cells and Globlet Cells Count

The Weibel method [22] was used to count mast cells and goblet cells, where the square area assigned to the cell count in the Weppel lens was estimated by balancing or calculating the area with the measurement segment of 1 millimeter length at X200 and then estimate that area, by estimating the number of points that exist above the tissue to count the cells within the counting box in the special lens and by the number of cells to be estimated, the percentage or number of cells required in the millimeter of tissue can be shown.

2.5 Determination of Paneth Cells Count

The pink-colored Paneth cells that appear in the bases of the tissues of the small intestine can be counted in about ten crypts, and then their arithmetic mean is taken and displayed in the sum of cells in the crypt base.

2.6 Determination of Internal Organs Weight

Internal organs were taken from all animals with different treatments such as heart, liver and kidneys and their weight was recorded after removal of impurities to observe the effect of the various concentrations of MTBE on organ weights.

2.7 Statistical Analysis

For statistical analysis of results, a one way Analysis of Variance (one way ANOVA), using SPSS version 17 for Windows [23].

3. RESULTS

The study showed that the different concentrations of MTBE affected the weight gain in general. The percentage increase in the weight ratio is lessen with increasing MTBE concentration in the water except for the concentration (2500 ppm) was higher than the first concentration and where the increase in weight for the animals in the control group was about 13.2%. On the contrary, weight increase in animals with high concentrations of MTBE which was almost 3% (Fig. 1). As observed animal groups treated with MTBE, as the concentrations of the compound increased, there was an increase feeling of thirst and consequently frequency of water consumption was evident.

The primary focus of the study was on estimating the quantitative changes in three important immune cells in the small intestine area of the treated mice. The results of the experiment showed that there was a significant change in the number of Mast cells, where the number of cells increased in the intestinal tissues as the concentration of MTBE in drinking water increased (5000 ppm) (Fig. 2). One-way analysis of variance (ANOVA) showed that there were significant differences in the number of mast cells among treated animals (P <0.002). Fig. 3-A shows the normal number of mast cells in the intestinal tissues of the mice, which are few in number, whereas in Fig. 3-B, the cells number has increased significantly in the treated animals.

The results show that the number of follicular mucous cells (Goblet cells) has increased (Fig. 4) from approximately 20 cells in mm^2 of mucous tissue in the intestinal tissues of untreated mice (Fig. 5-A) to approximately 65 cells per mm^2 at a concentration of 2500 ppm (Fig. 5-B), These findings were confirmed statistically showing significant differences between the number of goblet cells between control and MTBE-treated groups (P <0.01).

Changes in the counts of Paneth cells were also observed. The results presented in (Fig. 6) showed that the number of cells increased in the treated groups of mice compared to the control group. Fig. 7-A shows the number of Paneth cells in the intestinal tissues of the control mice while Fig. 7-B shows the number o Paneth cells in the treatment groups. Statistically significant differences in the counts of Paneth cells were observed between the control and the treated groups (P <0.01).

In addition to the effects of MTBE on immune cells counts, changes of the weights of some internal organs such as the heart (Fig. 8), kidneys (Fig. 9) and liver (Fig. 10) were noted. It is clear from these graphs that the weights of the internal organs increased in the animals treated with the MTBE compared to the control animals.

4. DISCUSSION

Since tissues in the small intestine are an area of absorption of nutrients and fluids, they are highly susceptible to the entry of any compounds that

are dissolved in the fluids into the body. Within this route, it is likely that MTBE could enter the blood stream directly from the small intestine and then with accumulative chronic or sub-chronic exposure to this compound adverse effects on the body could lead to the formation of various types health effects, such as liver and kidney dysfunctions; direct effects on nervous system as well as immune toxicity and finally formation of cancer which is believed to be mediated by the accumulation of α-2-globulin [11,24].

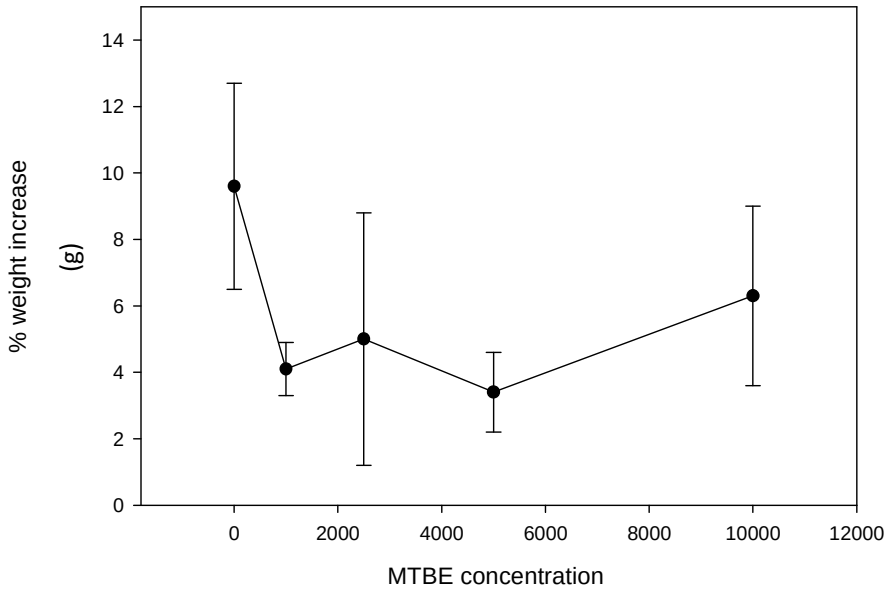


Fig. 1. Percentages of weight loss (in grams) differences between the control group (0.0 concentration) and MTBE-treated groups (mean ± standard error)

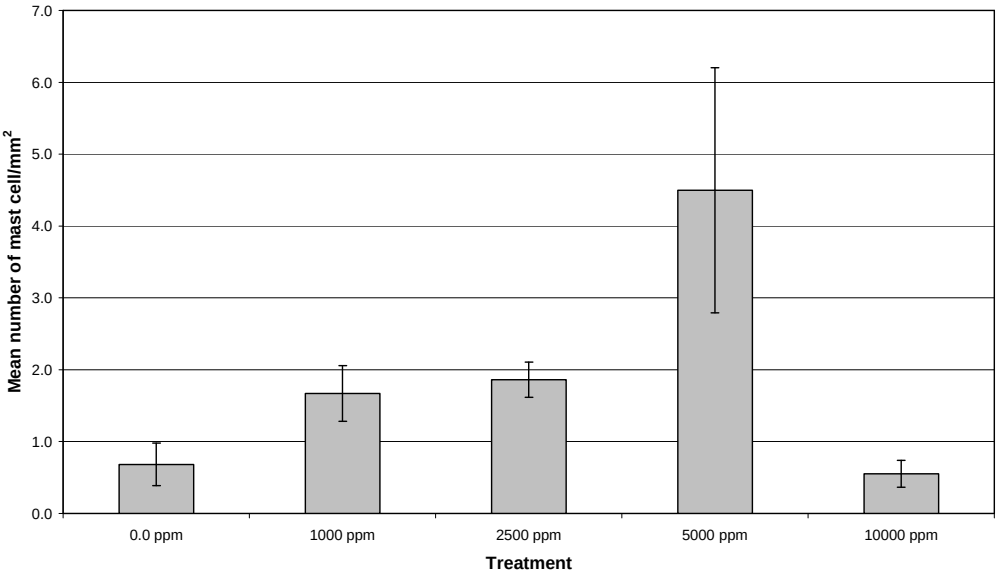
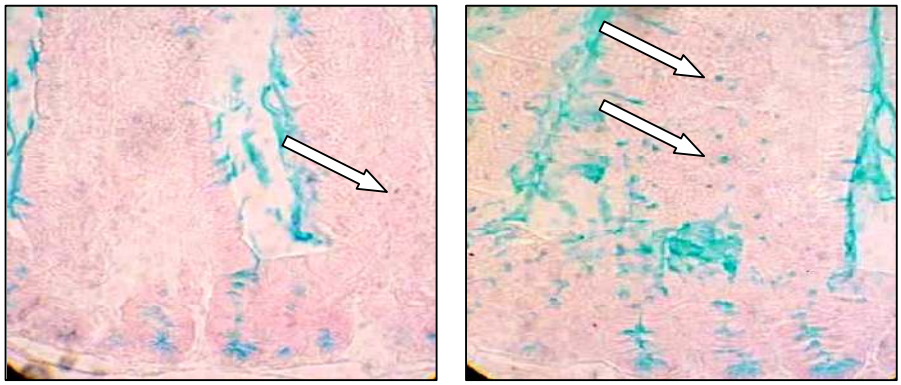


Fig. 2. The effects of MTBE on the counts of mucosal mast cells (0.0 ppm represents the control group), statistical significant difference was found (*P* < 0.002 - ANOVA) was found between untreated and treated animals (mean ± standard error)



3-A 3-B

Fig. 3. The number of mucosal mast cells in control group is obviously less (3-A) than the number of the cells in the MTBE treated group (concentration 5000 ppm) (3-B)

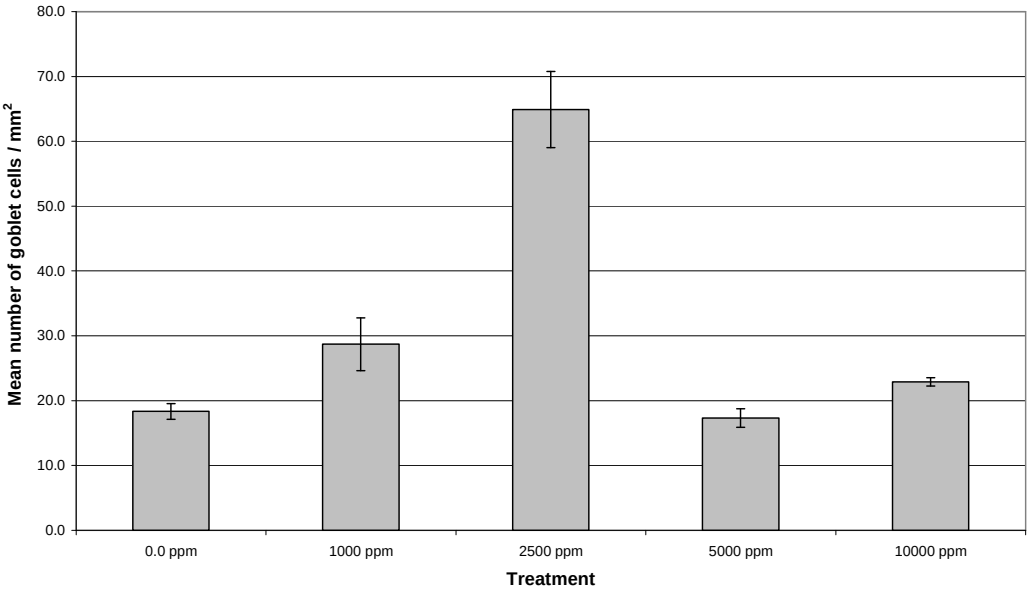


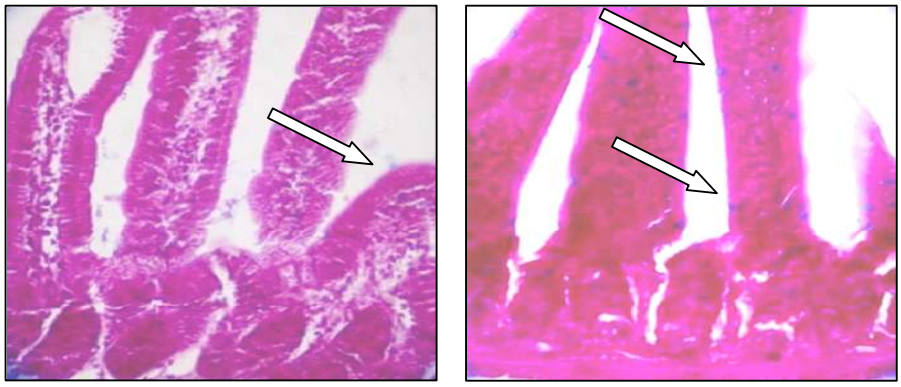
Fig. 4. Effects of MTBE on mucosal goblet cells counts (0.0 ppm represents the control group), statistical significant difference was found (*P* < 0.002 - ANOVA) was found between untreated and treated animals (mean ± standard error)

One notable observation was recorded throughout the experiment that the MTBE-treated mice had increased water consumption compared to the animals in the control group. This was coincided with frequent urination of the treated animals, particularly as the MTBE concentration increased; animals were frequently urinating and consuming drinking water. It was also noted that the odor of the urine of MTBE-treated mice was strong acetone-like, which perhaps explained by the fact that MTBE biotransformation in animals lead to the formation of acetone that is excreted in urine [10,25].

Mast cells play an important role in host defense immunity, particularly in mucosal tissues. The apparent increase of mast cells in the intestinal tissues of mice that were exposed to MTBE (Fig. 3) is probably as a response to the inflammatory effects of MTBE on intestinal tissues. As the concentration of absorbed MTBE increases, more mast cells were produced as inflammation mediators [26]. Mast cells release histamine and

other inflammatory mediators to help protects the mucosal tissues by enhancing mucosa production [27]. Mast cells may also induce other components of the immune systems to help protects these tissues against toxic chemical substances and pathogens. The number of mast cells increases in the sites of tissue injuries [26], perhaps chronic exposure to higher concentration of MTBE causes damages and injuries to intestinal tissues or in triggering the formation of tumor [10] and this perhaps attracts the release of mast cells to eliminate the cause of tissue injury or create a chronic inflammatory response [26].

Paneth cells are known for their important roles in intestinal innate immunity particularly in regulating microbial density in the intestine and protecting nearby stem cells [28]. The numbers Paneth cells usually range between 5 to 12 cells in the small intestine crypts; this was observed in the intestinal tissues of the control animals, with exposure to MTBE in drinking water, the number of Paneth cells increased to more than 25 cells (Fig. 6). Increase of Paneth cells occur as a result of microbial invasion, as well as in response to tissue injuries as a result to any inflammatory precursor [29]. It is possible that the inflammation on intestinal tissues induced by



5-A **5-B**

Fig. 5. The number of goblet cells is lower in the intestinal tissues of the control group (Fig. 5-A) compared to the increasing number of the cells in treated groups (2500 ppm) (Fig. 5-B)

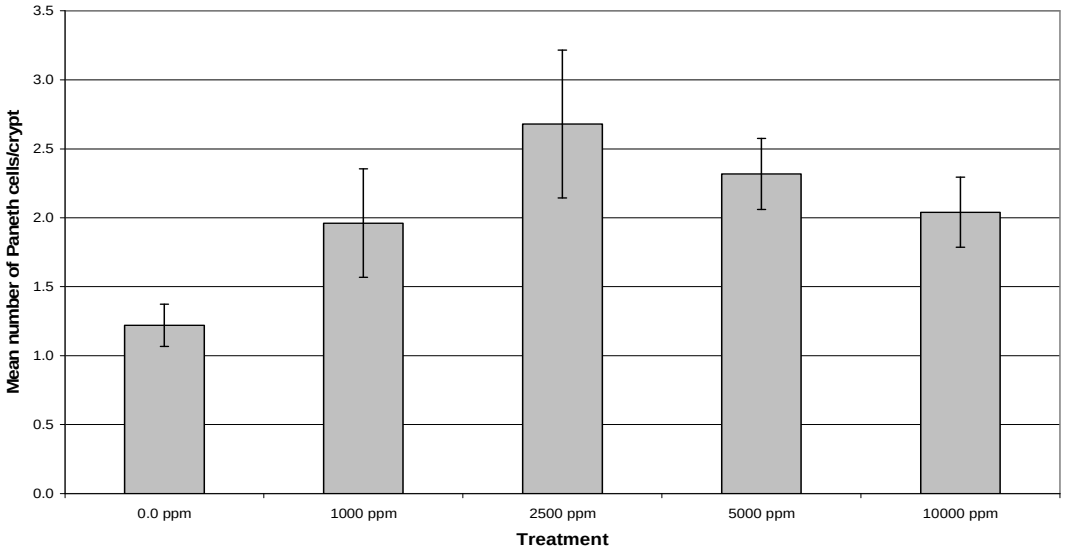
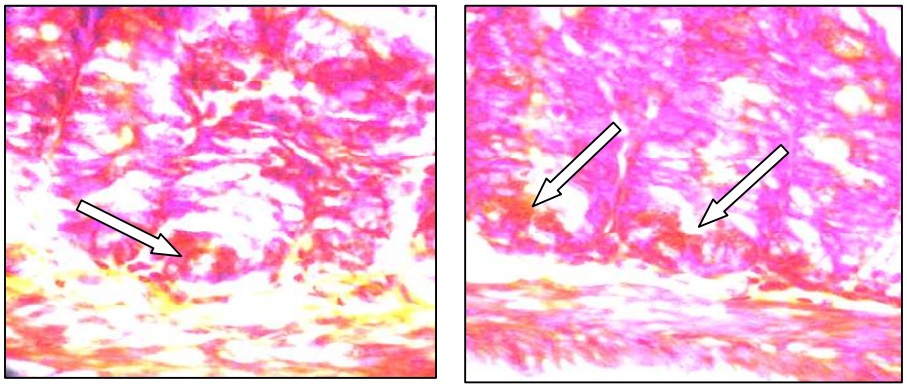


Fig. 6. The effect of MTBE on the counts of mucosal Paneth cells in control groups (0.0 ppm) compared with the treated groups, statistical significant difference was found ($P < 0.002$ - ANOVA) was found between untreated and treated animals (mean ± standard error)



7-A 7-B

Fig. 7. The normal number of Paneth cells in control mice (7-A), compared to the increased number of the cells in the treated mice (7-B)

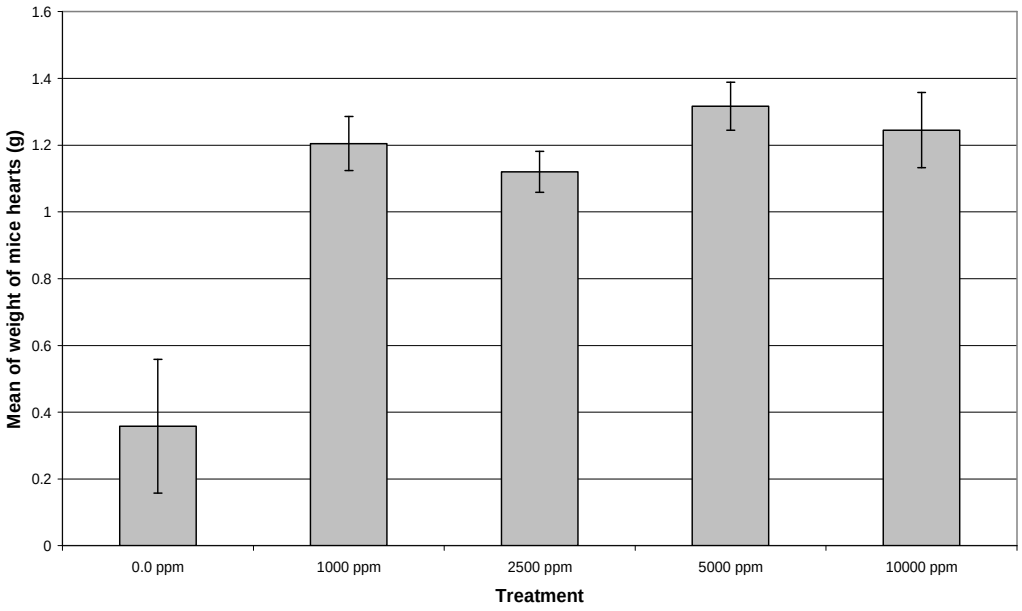


Fig. 8. The effects of MTBE on the weight of heart of treated mice compared to the control group (0.0 ppm) (mean ± standard error)

exposure to MTBE in treated mice played a role in the increase of the numbers of Paneth cells as a protective response.

The increased number of Paneth cells released in the intestinal crypts as a result of exposure to MTBE at concentration of 2500 ppm (Fig. 7) coincided with increase of goblet cells at the same concentration (Fig. 4 and Fig. 5). As a result of the possible tissue injury due to MTBE exposure more goblet cells are released to produce more mucins to lubricate and restore the damages of the intestinal mucous membranes [30].

It was also observed that MTBE has affected the weight of internal organs of treated animals. weight of heart, liver and the kidneys were notably all increased in animals consumed MTBE-contaminated drinking water compared to animals drinking MTBE-free water (Figs. 8, 9, 10). Increase of organs weights (liver and kidney) due to exposure to various concentrations of MTBE (3000 – 8000 ppm) in experimental animals (mice and rats) was reported [31,32]. It is assumed that the increase of organs weight is due to physiological changes, given that MTBE metabolized in liver, and also due to induction of renal and hepatic tumors by producing alpha-2-

globulin and defects in estrogen metabolism respectively [32]. It was also noted that animals treated with MTBE had sharp decrease in their weight gain (Fig. 1). Decrease of weight-gain in animals exposed to MTBE has been reported in various studies, this observed decrease of weight-gain due to exposure of MTBE was recently hypothesized as direct link between MTBE exposure and effects on endocrine system in mammals [33]. No exposure-related deaths were observed in this study.

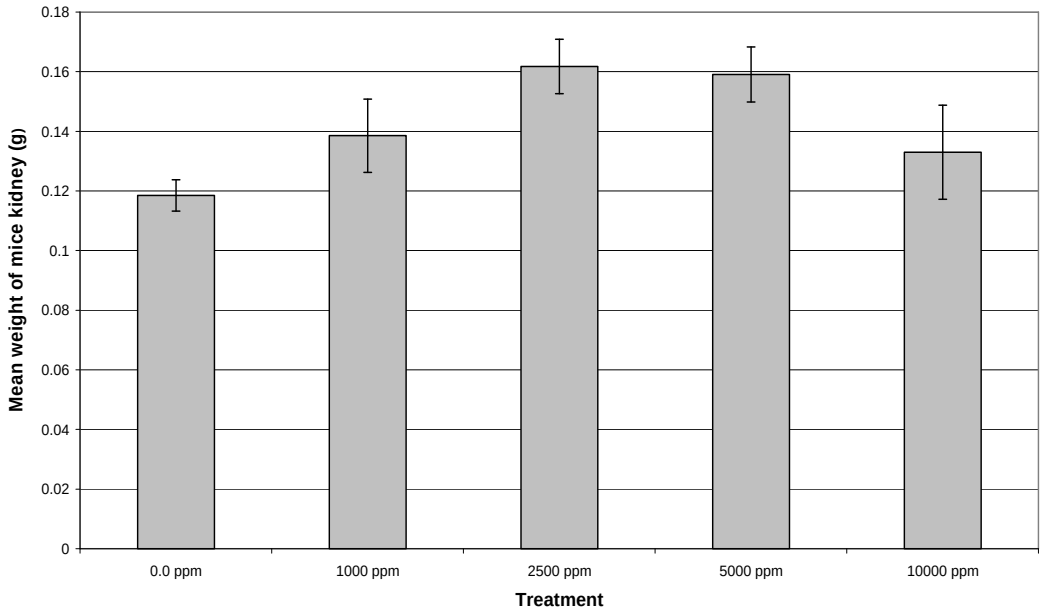


Fig. 9. The effects of MTBE on the weight of kidney of the treated mice compared to the untreated individuals (0.0 ppm) (mean ± standard error)

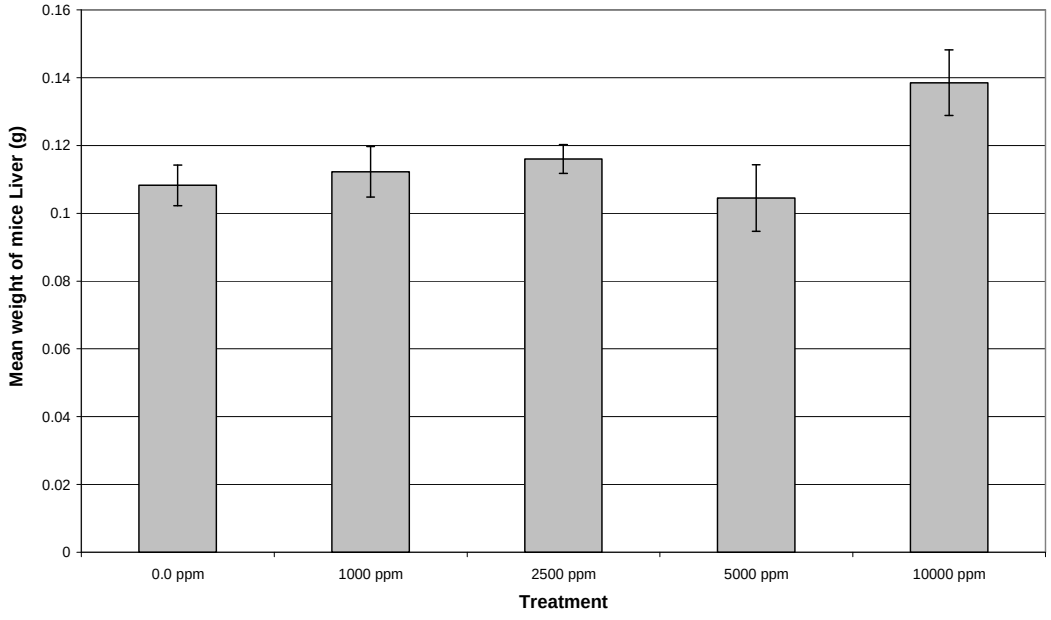


Fig. 10. Effects of MTBE on the weight of liver in treated animals compared to the untreated individuals (0.0 ppm) (mean ± standard error)

5. CONCLUSION

In conclusion The chronic exposure of intestinal tissues to MTBE may led to inflammatory effects and /or tissue damages, and as a response the mucosal innate immunity, particularly the numbers of mast, Paneth and goblet cells were increased in synergistic fashion to play their protective role in lessen the damages caused by MTBE.

ETHICAL APPROVAL

The experiments were performed in accordance with the guidelines for the care and use of laboratory animals of Umm Al-Qura University, Faculty of Applied Science, Institutional Animal Care Use Committee (IACUC), Saudi Arabia.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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