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Physiological and Histological Studies on the Effect of Melittin on Mice Jejunum

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Melittin is a major polypeptide in honey bee venom that has been used traditionally against chronic inflammation and cancer. The aim of this work is study the effect of melittin on the mice jejunum from the physiological and histological points, then determine the safe dosage and duration on cells and tissues. Adult male mice (Albino Swiss) were divided into two groups (7 mice for each group): control group and melittin only treated group (10 and 40 μg/kg). The samples from the jejunum were collected and prepared for histological and physiological studies. Melittin has no cytotoxic effects, where the alcian blue stained sectioned showed the normal distribution of the mucous secreting cells and the levels of IL-1b in the jejunum mice treated with melittin (10 or 40 μg/kg) for 3 or 5 days were closed to control, whereas these levels were significantly decreased compared to control after treated for 10 days for each doses. These studies suggest that the using of melittin in small doses and short duration were safe and don't cause any toxic effects on the jejunum tissue. Therefore, melittin might be effective in treatment of gastrointestinal diseases.

Key words: Melittin, gastrointestinal tract, IL-1b.

Honey bee venom (apitoxin) is a bitter colorless liquid. The active portion of the venom is a complex mixture of proteins that causes local inflammation and acts as an anticoagulant. There are at least 18 active components in the venom that have some pharmaceutical properties[1, 2]. Bee venom includes melittin, apamin, adolapin, the mast-cell-degranulating (MCD) peptide, enzymes (phospholipase A2), and biologically active amines (histamine and epinephrine), aside from its non-peptide components[2].

Melittin has been used as anti-inflammatory and anti-cancer drug[3]. It is a 26-residue bee venom peptide that folds into amphipathic á-helix and causes membrane permeabilization via a mechanism that is still disputed[4] and induces various reactions in membranes and has been widely studied as a model for membrane-interacting peptide[5]. It is a strong anti-inflammatory agent and induces the production of cortisol in the body. It is also a cell-lytic agent[2]. Previously it found that melittin played a central role in the production of nociceptive responses and cutaneous hypersensitivity after whole bee venom injection[6, 7].

In addition, melittin has broad-spectrum, fast-acting and highly effective inhibitory effects on both pathogenic and agricultural microorganisms, which demonstrated its application potential as a biological pesticide[8].

On other hand, melittin has been widely used in the treatment of some immune-related diseases, as well as in recent times in treatment of tumors. Several cancer cells, including renal, lung, liver, prostate, bladder, and mammary cancer cells

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as well as leukemia cells, can be targets of bee venom peptides such as melittin and phospholipase A2. Therefore, It has ability to induce cytotoxic, antitumor, immunomodulatory, and apoptotic effects in different tumor cells *in vivo* or *in vitro*[9].

The aim of this research is to study the effect of melittin on mice jejunum from the physiological and histological sides and to determine the safe dosage and duration on jejunal cells and tissues.

MATERIALS AND METHODS

Melittin

Melittin (the principle hemolytic component of honeybee venom) was obtained from Sigma Chemical Company in the form of powder that exhibited a purity of e"85% by HPLC. 0.135 grams of melittin was dissolved in 100 ml of distilled water. Melittin solution was divided into small aliquots that kept frozen (-20°C) until the time of use. The solution was diluted to prepare the required concentrations (10 and 40 µg/kg body weight).

Experimental animals

Adult male Albino mice (25±5 g) were kindly supplied by The Animal House of King Fahd Medical Research Center, King Abdulaziz University, Jeddah. The mice were transferred to wire-bottomed cages at the animal house of King Fahd Medical Research Center. The animals were kept at an ambient temperature and fed on a special rodent diet supplied by Medical Professions for Veterinary Products and Fodders Additions Company (MUVCO). The mice were given fresh water through glass bottles with a capillary dropper fixed to the wall of the cage in a position to be available for the mice. Water was changed and the cages were cleaned every day. The mice were weighted just before the beginning of each experiment.

Experimental groups

Control group

The control group included seven adult male Albino mice. Each mouse was treated by using the stomach feeding tube with a daily dose of 1 ml distilled water for ten days.

Melittin group

Forty two mice were divided into six subgroups (7 mice each) and treated by using the stomach feeding tube as follows: The first three subgroups were treated daily with a melittin (10 µg/kg body weight) for 3, 5 or 10 days, while the others were treated daily with a single dose of melittin (40 µg/kg body weight) for 3, 5 or 10 days[10].

After 24 hours from each treatment, mice of all groups were sacrificed under light ether anesthesia. Samples from the jejunum collected from all animals were prepared for physiological, histological and immunohistochemical studies.

Physiological studies

Physiological studies were performed using Enzyme-Linked Immunosorbent Assay (ELISA) kits (obtained from USCNK company) to determine the release of cytokine (IL-1B) in the jejunum of control and experimental groups. ELISA procedure that used in the present study was according to the method of Moreels *et al.* [11]; Abdu and Alahmari[12].

Data analysis

The concentrations of the cytokine (IL-1B) of the jejunum mucosa isolated of the experimental groups were compared with the concentrations of these agents of the mucosa isolated from the control mice. Data was expressed as the mean of concentration ± SE) standard error), with *n* being the number of animals. Statistical significance was measured by *t*-test using SPSS software and was designated at the level of $P< 0.05$.

Microscopic studies

On scarification, samples from the body of the jejunum were immediately removed from each animal and then washed within a physiological saline solution (0.85% NaCl) for the removal of the blood or food remnants, which might obstruct the process of fixation. Small pieces (about 4 mm in diameter) from each sample were obtained by using a sharp blade. Tissue samples were allowed to remain in the fixative (10% neutral buffered formalin) for 24 hours. The fixed samples were washed in running water for overnight, then dehydrated through ascending series of ethyl alcohol (30%, 50%, 70%, 80%, 90%, 95%, and 2 changes of 100%) 2 hours each.

Clearing was next by moving the tissues into a mixture of absolute ethanol and toluene (1:1) for 2 hours, then in two changes of pure toluene (2 hours each). Tissue samples were then placed into

a mixture of toluene and paraffin (1:1) at the oven. The tissues were then infiltrated in pure paraffin and embedded in paraffin block by using Paraffin Embedding Machine (LS-100; Bio-Equip Company). The blocks were allowed to cool slowly in a water bath (20-25°C).

Paraffin blocks were trimmed for removing excess paraffin around the tissues sample by using sharp blade. The paraffin blocks were sectioned at a thickness of five microns by using rotating microtome (Bright instrument LTD, England) at the Histology Unit of Anatomy Department, Faculty of Medicin, King Abdul-Aziz University. The paraffin sections were floated over a warm water bath and picked up by clean glass microscopic slides, which contained glycerin Mayer's adhesive media (egg albumin + glycerin + sodium salicylate). The slides were placed on a warm oven at 25°C for about 15 minutes. The paraffin sections were used in the following techniques:

Alcian blue technique

Alcian blue staining technique was used to detect acid muco-substances and acetic mucins in different mucous secreting cells of the jejunum[13, 14].

Immunohistochemical (IHC) techniques

Immunohistochemical staining is a valuable tool for detecting specific antigens in tissues[15]. The Epithelial Membrane Antigen (EMA) (obtained from Ventana Company) was used in the present study to detect epithelial cell mouse monoclonal antibody.

RESULTS

Physiological results

IL-1B Concentration of jejunum

Control group

The mucosal IL-1B concentration of control jejunum was 167±3 pg/ml, (n=7). This value was used to compare with experimental groups to determine the effect of melittin on jejunum tissues.

Melittin treated group

Effect of melittin (10 ±g/kg)

IL-1B concentration in the mucosal jejunum of mice treated with melittin (10 µg/kg) for 3 or 5 days was nearly similar to control (163±4 and 170±4 *vs* 167±3 pg/ml, P> 0.85 and 0.33 respectively, n=7). However, after 10 days IL-1B concentration was decreased significantly compared to control (138±4 *vs* 167±3 pg/ml, P< 0.03, n=7) (Fig. 1, Table 1).

Effect of melittin (40 ±g/kg)

The level of IL-1B in the mucosal jejunum of mice treated with melittin (40 µg/kg) for 3 or 5 days was closed to control (151±4 and 172±4 *vs* 167±3 pg/ml, P> 0.08 and 0.6 respectively, n=7), whereas IL-1B concentration in mice treated by melittin (40 µg/kg) for 10 days was significantly decreased compared to control (142±4 *vs* 167±3 pg/ml, P< 0.04, n=7) (Fig. 2, Table 2).

Microscopic results

Alcian blue-stained sections

Alcian blue stain was applied for the detection of the goblet cells and the mucous secreting cells in the Brunner's glands of the control and experimental groups. The nuclei of all cells in the jejunum sections were counterstained blue with hematoxylin.

Control group

Control sections of jejunum tissues showed normal distribution of blue stained goblet cells that lie between the mucosal columnar epithelial cells of the jejunum villi (Fig. 3A). At the submucosa, the mucous secreting cells of the Brunner's glands were also stained with blue coloration which reflects their mucous contents (Fig. 3B).

Melittin treated group

The jejunum tissues of mice treated with

Table 1. Mean values of IL-1B concentration ± SE in pg/ml in mucosal tissues of the jejunum in melittin group (10 µg/kg) compared to control group. $^{*}P$< 0.05. Paired-samples t-test, n=7

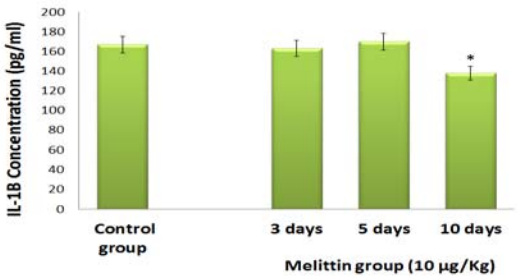
Melittin Group 10 µg/kg			Control	Groups
10 days	5 days	3 days	Group	
138±4*	170±4	163±4	167±3	IL-1B Con.

Table 2. Mean values of IL-1B concentration ± SE in pg/ml in mucosal tissues of the jejunum in melittin group (40 µg/kg) compared to control group. *P< 0.05. Paired-samples t-test, n=7

Melittin Group 40 µg/kg			Control	Groups
10 days	5 days	3 days	Group	
142 ± 4*	172 ± 4	151 ± 4	167 ± 3	IL-1B Con.

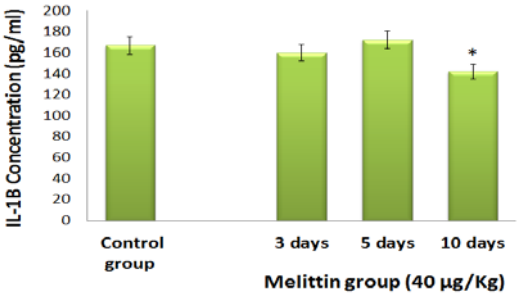
10 µg/kg melittin for 3 days showed the positively stained mucosal goblet cells and the mucous secreting cells of the Brunner's glands (Fig. 4A), which were more or less similar in their distribution and Alcian blue stainability to those of the control sections. Normal distribution of goblet cells and mucous secreting cells of the Brunner's glands were also recorded in jejunum tissues of mice treated with the same dose of melittin for 5 days (Fig. 4B). After treatment with the same dose of melittin for 10 days, the goblet cells showed their common positively stained appearance. However, only few positively stained mucous secreting cells of the Brunner's glands were noticed in some areas of the jejunum tissues of mice treated for 10 days (Fig. 4C).

Mice treated with 40 µg/kg melittin showed normal pattern of the distribution and stainability of the mucosal goblet cells after 3 days (Fig. 4D), 5 days (Fig. 4E) or 10 days (Fig. 4F) of treatment. However, only a few mucous secreting cells of the Brunner's glands were positively



Note that IL-1B concentration in the mucosal jejunum of mice treated with melittin (10µg/kg) for 3 and 5 days was nearly similar to control. However, after 10 days IL-1B concentration was decreased significantly compared to control. $^{*}P< 0.05$.

Fig. 1. Histogram showing the concentration of IL-1B in melittin group (10µg/kg) compared to control group



Note that the level of IL-1B in the mucosal jejunum of mice treated with melittin (40 µg/kg) for 3 and 5 days was closed to control, whereas IL-1B concentration in mice treated with melittin (40 µg/kg) for 10 days was significantly decreased compared to control. $*P< 0.05$.

Fig. 2. Histogram showing the concentration of IL-1B in melittin group (40µg/kg) compared to control group

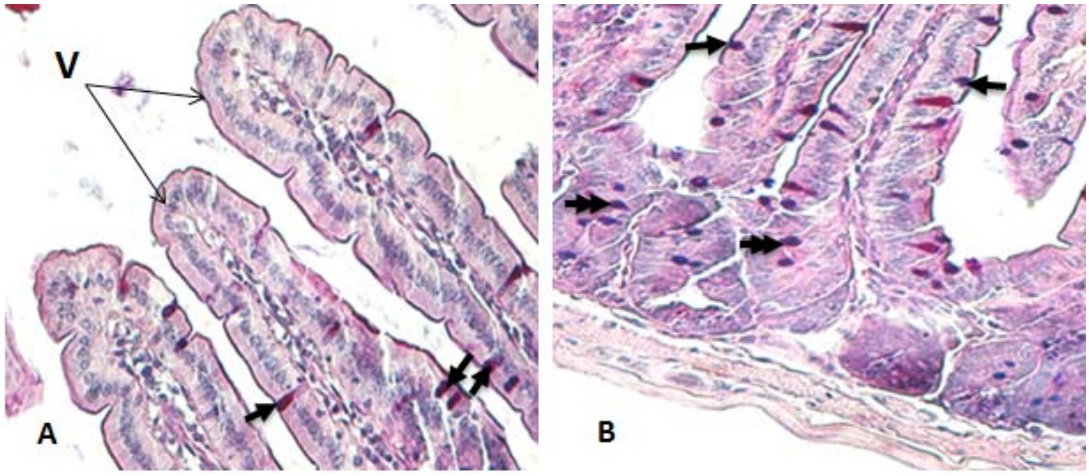
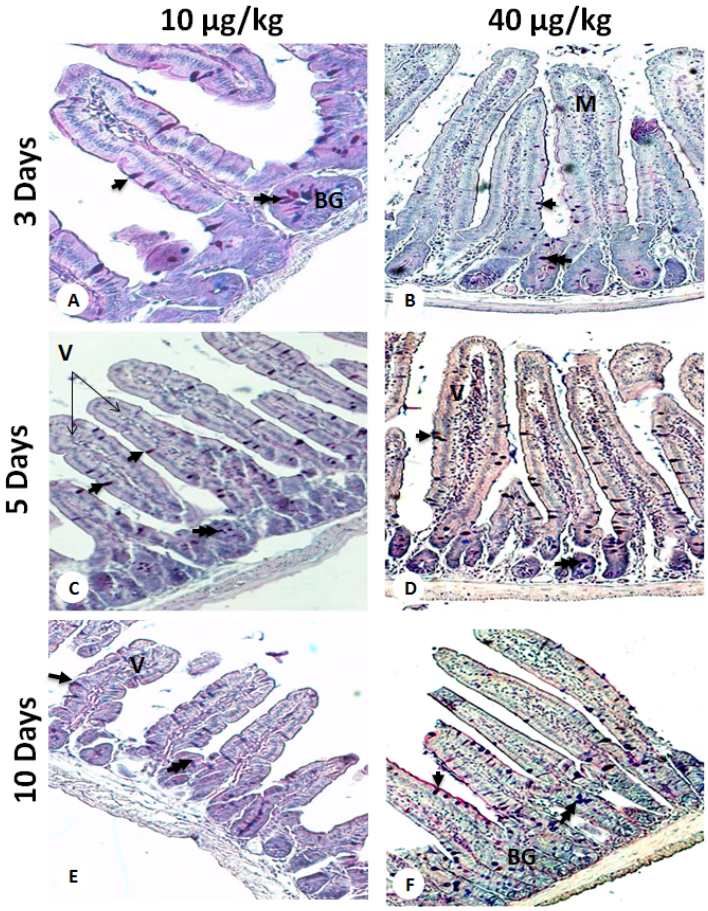
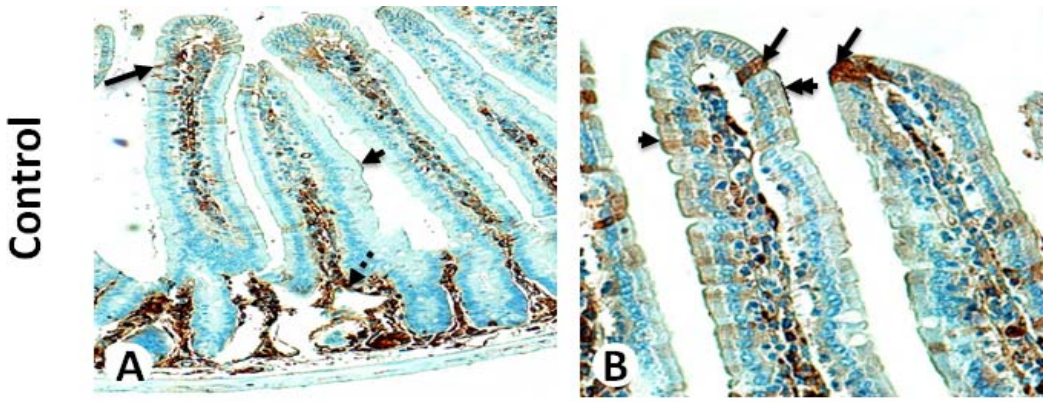


Fig. 3. Alcian blue stained sections of the jejunum in the control mice **(A)** showing villi (V) with normal distribution of goblet cells (arrows) that positively reacted with Alcian blue (Alcian blue, X100). **(B)** Positive reactivity with Alcian blue at the mucosal goblet cells (arrows) and the mucous secreting cells of the Brunner's glands (double arrows) (Alcian blue, X400)



Note the common distribution of the goblet cells (arrow) and the mucous secreting cells (double arrows) of the Brunner's glands (BG) that positively reacted with Alcian blue (Alcian blue, X100)

Fig. 4. Effects of melittin on the jejunum tissue. Alcian blue stained sections of the jejunum after treated with 10 µg/kg melittin for 3 days (A), 5 days (B) or 10 days (C); and 40 µg/kg melittin for 3 days (D), 5 days (E) and 10 days (F)



Note a few mucosal epithelial cells reveal intense EMA activity at the whole cell (arrow) (EMA immunohistochemistry, X100, X400).

Fig. 5. Transverse section in the jejunum of **(A)** and **(B)** a control mouse showing moderate (arrow head) to intense (double arrow heads) along the luminal cell membrane of the mucosal epithelial cells that cover the mucosal villi

stained with Alcian blue in all jejunum sections of mice treated with 40 µg/kg melittin compared to those of the control group.

Immunohistochemical reactivity of the epithelial membrane antigen (EMA)

The immunohistochemical reactivity of the epithelial membrane antigen (EMA), the specific antigen of the cell membrane of the epithelial cells was indicated by brown coloration at the epithelial cells of the jejunum tissues of control and treated mice.

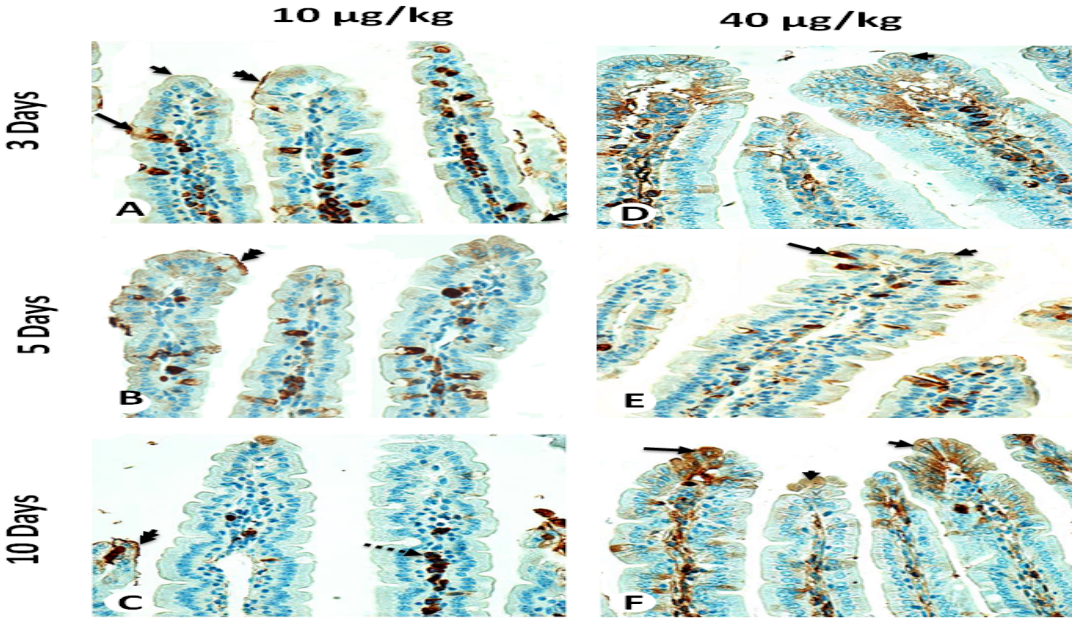
Control group

The jejunum tissues of control mice showed moderate to intense EMA reactivity at the luminal (apical) cell membranes of the mucosal epithelial cells that cover the mucosal villi. A few mucosal epithelial cells also showed intense reactivity at the whole cell. Moreover, the epithelial lining cells of the gastric glands showed negative EMA reactivity. The lymphocytes at the lamina properia displayed intense reactivity (Fig. 5A-B).

Melittin treated group

The jejunum tissues of mice treated with 10 µg/kg melittin for 3 days (Fig. 6A) showed moderate to intense EMA reactivity at the luminal cell membranes of the mucosal epithelial cells, besides intense EMA reactivity a few whole cells. Similar EMA reactivity was displayed in the mucosal epithelium of the jejunum tissues of mice treated with the same dose of melittin for 5 days (Fig. 6B) or for 10 days (Fig. 6C). The epithelial lining cells of the gastric glands showed negative EMA reactivity, while the lymphocytes at the lamina properia revealed positive reactivity in all tissues of mice treated with 10 µg/kg melittin.

After treatment with 40 µg/kg melittin for 3 days (Fig. 6D), the jejunum tissues of the mice showed moderate EMA reactivity at the luminal



EMA immunohistochemistry stained sections of the mice jejunum treated with alone melittin (A) 10 µg/kg/3 days showing moderate (arrow head) to intense (double arrows) EMA reactivity at the mucosal epithelium. Note a few whole cells that display intense reactivity (arrow); (B) 10 µg/kg/5 days showing enlarged part of the mucosal epithelium that display to moderate (arrow head) to intense (double arrow) EMA reactivity; (C) 10 µg/kg/10 days showing intense EMA reactivity at the luminjal cell membranes of some mucosal epithelial cells (double arrow heads). Dotted arrow indicate intense EMA reactivity of the lymphocytes at the lamina properia; (D) 40 µg/kg/3 days showing moderate reactivity at the luminal cell membranes of the mucosal epithelial cells (arrow head); (E) 40 µg/kg/5 days showing few mucosal epithelial cells (arrow) with intense EMA reactivity. Note moderate reactivity at the luminal cell membranes of the mucosal epithelial cells; (F) 40 µg/kg/10 days showing the mucosal epithelium reveal intense EMA reactivity (arrow), while others reveal moderate (arrow head).

Fig. 6. Effect of melittin on the jejunum

cell membranes of the mucosal epithelial cells. Moderate EMA reactivity was also exhibited by the luminal cell membranes of the mucosal epithelial cells in the jejunum tissues of mice treated with the same dose for 5 days (Fig. 6E) or for 10 days (Fig. 6F). Moreover, a few mucosal epithelial cells showed either intense or moderate EMA reactivity at the whole cell in the jejunum tissues of mice treated for 5 or 10 days with the same dose of melittin. The Brunner's glands in all melittin treated mice showed negative reactivity.

DISCUSSION

The level of IL-1B was nearly normal in the mucosal jejunum of mice treated with melittin (10 and 40 µg/kg) for 3 or 5 days, but significantly reduced after treatment for 10 days. The prolonged administration of melittin could be the reason for the observed reduction of IL-1B concentration when treated for 10 days (16). This result differ from the results of Yun *et al.*[10] on pancreas, who reported that melittin did not have any effect on the cytokines level of healthy mice. Our justification for that is the reduction could be due to short duration of treatment (twice injection with 10-50 µg/kg of melittin) while in this study the mice were treated with 10- 40 µg/kg of melittin for 3, 5 and 10 days.

The IL-1B reduction also supported by Khan and Ghia[17], they mentioned that there is close proximity between enterochromaffin cells and immune cells in the mucosa of GI tract.

On the other hand, alcian blue stained sections of the jejunum after treated with 10 or 40 µg/kg melittin for 3, 5 or 10 days showed the common distribution of the goblet cells and the mucous secreting cells of the Brunner's glands that positively reacted with Alcian blue. These results confirmed with several studies on intestine and pancreas, where these studies demonstrated that low concentrations of melittin did not have any harmful effects on the tissues and cellular functions such as mucus secretion[18, 10].

In the present study, the immunohistochemical techniques were used to determine the Epithelial Membrane melittin treated mice, where EMA (or MUC1) is the major component of the apical surface of the epithelial cells, which contains a hydrophobic stretch of amino acid residues anchoring the long filamentous molecules in the plasma membrane[19]. This antigen creates a highly hydrophilic region which prevents hydrophobic chemotherapeutic drugs from passing through the cell surface, thus preventing the drugs from reaching their targets which usually reside within the cell; therefore, it provides a kind of protection for the cells[19]. On other hand, no changes were observed on the reactivity of EMA in tissues of mice treated with melittin confirmed the safety effects of melittin to ameliorate the apical cell membranes of the mucosal epithelial cells.

In conclusion, our results suggest using melittin as a cure for certain period will be safe on the jejunum tissue.

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