Protective Effect of Melittin against Gastric Inflammation in Mice

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Abstract: The major compound of bee venom, melittin, has been used as an anti-inflammatory reagent for decades. However, the potential of melittin to ameliorate stomach inflammation is unknown. Our aim was to investigate the effect of melittin on indomethacin-induced gastrointestinal inflammation. Adult male Albino mice (Swiss mice strain) were randomly divided into four groups (7 mice each group): control group; indomethacin-treated group (50 mg/kg) for 1day; melittin treated group (10 or 40 µg/kg) for 3,5 or 10 days; and melittin/indomethacin treated group. The results of the histological studies showed that the effect of indomethacin on the stomach tissue of mice included superficial erosion and exfoliation of some epithelial cells to the gastric lumen, also large areas full of numerous inflammatory cells were seen at the submucosa and extend to different parts of the lamina properia. Besides the depletion of antibody of epithelial membrane antigen (EMA) in the stomach tissue. In accordance with the results of *in vivo* experiments, melittin doses (10 and 40 µg/kg) inhibited histological and immuonohistochemical changes in the stomach during inflammation induced by indomethacin, where the gastric tissues showed more or less intact mucosal epithelial cells and the submucosal inflammatory cells were less in number compared to those recorded in the gastric tissues of indomethacin-treated mice. Also normal histological structures of the gastric glands, the muscularis mucosa, the muscularisexterna and the serosa were recorded. On other hand, it was showed the reactivity of EMA in the stomach tissues were reduced under the effect of indomethacin treatment, while melittin restored the reduction in EMA reactivity induced by indomethacin in tissues, this observed could be attributed to the protective effect of melittin against the abnormality cases of epithelial cells. In conclusion, these results clearly indicate that melittin provided protection against indomethacin-induced gastrointestinal inflammation through its ability to protect the epithelial lining cells of the stomach by suppressing the activity of phospholipase and protease enzymes which may contribute to the exfoliation and erosion of the mucosal epithelial cell.
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1. Introduction

Inflammation is a homeostatic response expectedto limitaccess of foreign substances to the body and of facilitating repair (Martin and Wallace, 2006). In the gastrointestinal (GI) tract, the mucosa is continuously challenged by a variety of aggressive factors of both endogenous and exogenous irritants, including excess secretion of gastric acids and pepsin, ethanol, reactive oxygen species, non-steroidal anti-inflammatory drugs (NSAIDs), excess psychiatric stress, and *Helicobacter pylori* (*H. pylori*) infection (Choi *et al*.,2009). The inflammatory process is a key component of mucosal defense against exogenous and endogenous factors. Impairment of this response can lead to mucosal injury and to destruction of repair processes. The dysregulated inflammatory responses can deterioratedamage in the GI tract and contribute to the generation of symptoms. The inflammatory response is coordinated by a range of mediators that are released from the epithelium and from cells within the lamina propria (e.g., mast cells, lymphocytes, neurons, and fibroblasts) (Martin and Wallace, 2006).

NSAIDs, such as indomethacin, are the most commonly prescribed drugs worldwide, which attest to their efficacy as analgesic, antipyretic and anti-inflammatory agents as well as anticancer drugs(Wallace, 1997; Choi *et al*., 2009).Using a high dose of indomethacin contributes to gastric ulcer and GI inflammation (Olaleye*et al.*, 2013). The development of "coxib" (selective cyclooxygenase-2 inhibitors) offered similar efficacy with reduced toxicity. Mechanistically all of these adverse outcomes with NSAID use are strongly related to the impairment of integrity maintenance in the gastroduodenal mucosa (Wallace, 1997; Choi *et al*., 2009).

The treatment of gastrointestinal inflammation by using natural products was reported by several investigators. In this concern, Fawole*et al*. (2009) reported the possibility of using twelve different medicinal plants in treating pain and cramp related to gastro-intestinal tract inflammation, where these plants contained phenolics, tannins, flavonoids, alkaloids and saponins which can inhibit the activity of COX enzymes. It was also found that

some materials isolated from marine organisms, like algae, were contributed in the treatment of both acute and chronic models of gastrointestinal inflammation (Aslam, 2010). In addition, Prakash*et al*. (2008) found that consuming the manuka honey could reduce gastrointestinal inflammation in rats, which might help to control or treat some types of ulcerative gastroenteritis.

Melittin has multiple effects, including antibacteria, antivirus, and anti-inflammation, in various cell types (Raghuraman and Chattopadhyay, 2007). Some studies have shown that melittin can induce cell cycle arrest, growth inhibition, and apoptosis in various tumor cells (Duke *et al*., 1994; Zhang *et al*., 2007).

The mechanism of melittin-induced activation has been described for phospholipid model membranes in an assay in which melittin was added in the form of tetramers (Hermetter and Lakowicz, 1986). The uses of melittin for treatment of inflammation in different tissues were reported by several investigators, such as the protective effect of melittin on inflammation induced in case of osteoarthritic chondrocytes (Nah *et al*., 2007), rheumatoid arthritis (Stuhlmeier, 2007; Li *et al*., 2010) acute pancreatitis (Yun *et al*., 2011) and acute liver inflammation (Park *et al*., 2012). However, limited studies have been focused on the effect of meittin on gastric inflammation induced by indomethacin in the mice.

2.Materials and Methods

Indomethacin

Indomethacin was used to induce inflammation in mice gastrointestinal tract. It was obtained from Sigma Chemical Company in the form of powder 0.027 grams of indomethacin was dissolved in 1 ml of 70 % ethanol and diluted by distilled water to 9 ml before use.

Melittin

Melittin was obtained from Sigma Chemical Company in the form of powder. 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).

Experimental groups

A- 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.

B- Indomethacin group

Seven mice were treated by using the stomach feeding tube, each with a single dose of indomethacin (50 mg/kg body weight) to induce gastrointestinal inflammation (Venkova*et al*., 2008).

C- Melittin group

Forty two mice were divided into six subgroups (7 mice each) and treated by using the stomach feeding tube as follows:

- Three subgroups were treated daily with a melittin (10 µg/kg body weight) for 3, 5 or 10 days.
- Three subgroups were treated daily with a single dose of melittin (40 µg/kg body weight) for 3, 5 or 10 days (Yun *et al*., 2011).

D- Indomethacin-Melittin group

Forty two mice were used to investigate the effect of melittin on indomethacin treated group. These mice were divided into six subgroups (7 mice each) as follows:

- Three subgroups were treated with indomethacin (50 mg/kg/1 day) followed by treated daily with a single dose of melittin (10 µg/kg body weight) for 3, 5 or 10 days.
- Three subgroups were treated with indomethacin (50 mg/kg/1 day) followed by treated daily with a single dose of melittin (40 µg/kg body weight) for 3, 5 or 10 days.

After 24hours from each treatment, mice of all groups were sacrificed under light ether anesthesia. Samples from the stomach body 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 Medicine, 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:

1- Hematoxylin and Eosin (H&E) technique:

H&E technique was used to investigate the histological pattern of tissues obtained from the stomach body of control and experimental groups.

2- Immunohistochemical (IHC) technique:

Immunohistochemical staining is a valuable tool for detecting specific antigens in tissues (Cuello, 1993). Anti-Polyvalent, HRP/DAB (Horseradish peroxidase/ Diaminobenzidine) kit was used as ultravision detection system which detects a specific antibody bound to an antigen in tissue sections. The technique used was streptavidin-biotin immunoenzymatic antigen detection system. It involved the sequential incubation of the specimen with an unconjugated primary antibody specific to the target antigen, a biotinylated secondary antibody that reacts with the primary antibody, enzyme-labeled streptavidin and substrate/chromogen (Diaminobenzidine; DAB).

The Epithelial Membrane Antigen (EMA) (obtained from Ventana Company) was used in the present study as primary antibody to detect epithelial cell mouse monoclonal antibody.

3.Results

1- Hematoxylin and Eosin (H&E) technique:

Sections of stomach body tissues were stained with H&E to demonstrate the histopathological alterations in experimental animals (Table 1).

A- Control group

The microscopic examination of the body of the stomach obtained from control mice showed the common histological features of the four layers of the stomach wall: mucosa, submucosa, mascularisexterna and serosa (Figures1A-B).

B- Indomethacin treated group

After 24 hours from indomethacin administration, the mucosal epithelium of the body of the stomach showed superficial erosion and exfoliation of some epithelial cells to the gastric lumen (Figure 1C). Large areas full of numerous inflammatory cells were seen at the submucosa and extend to different part of the lamina properia (Figure 1D).

C- Melittin treated group

The body of the stomach of mice treated with 10 µg/kg melittin for 3 days (Figure 2A), 5 days (Figure 2B) or 10 days (Figure 2C) showed the common characteristic features of the four gastric layers. The gastric mucosa showed intact lining and the connective tissues of the lamina properia and the submucosa were devoid of any inflammatory cells. Similar observations were noticed in sections obtained from the body of the stomach of mice treated with 40 µg/kg melittin for 3 days (Figure 2D), 5 days (Figure 2E) or 10 days (Figure 2F).

D- Indomethacin – Melittin treated group

The gastric tissues obtained from the body of the stomach of mice treated with indomethacin followed by treatment with 10 µg/kg melittin for 3 days showed fewer number of inflammatory cells in the submucosa compared to those observed in the indomethacin treated group. The tissues also showed the presence of a few exfoliated mucosal epithelial cells and small loci of erosion (Figure 3A). After 5 days of treatment with 10 µg/kg melittin to mice pretreated with indomethacin, the gastric tissues showed more or less intact mucosal epithelial cells except for the presence of erosion at a few points. Moreover, the submucosal inflammatory cells were less in number compared to those recorded in the gastric tissues of indomethacin-treated mice after treatment with the same dose for 5days (Figure 3B). On other hand, the stomach of a mouse treated with indomethacin followed by treatment with 10 µg/kg melittin for 10 days showed the normal appearance of the epithelial mucosal cells and disappearance of most of the inflammatory cells, but the presence of a very few lymphocytes at the submucosa (Figure 3C).

Microscopic examination of sections obtained from the body of the stomach of indomethacin-treated mice that treated with 40 µg/kg melittinfor 3 days showed the presence of mostly intact mucosal epithelial cells with complete absence of erosion together with the noticeable reduction in the number of inflammatory cells in the submucosa. The gastric tissues of the present group also revealed normal histological profile of the gastric glands, the muscularis mucosa, the muscularisexterna and the serosa (Figure 3D)

Mice pretreated with indomethacin followed by treatment with 40 µg/kg melittinfor 5 days showed the common features of the stomach layers with intact mucosal epithelium, normal gastric glands and muscularis mucosa and muscularisexterna as well as normal serosa. Obvious decreases in the occurrence of inflammatory cells together with the presence of a few submucosal lymphocytes cells were also noticed (Figure 3E).

After treatment with 40 µg/kg melittin for 10 days to mice pretreated with indomethacin, the gastric tissues of the body of the stomach showed complete disappearance of the inflammatory cells at the submucosa and the occurrence of intact mucosal epithelium and gastric glands (Figure 3F).

2- Immunohistochemical (IHC) technique 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 stomach body tissues of control and treated mice. EMA reactivity of different cells was summarized in table (2).

A- Control group

In control group, intense EMA reactivity was observed along the luminal cell membranes of the mucosal epithelial cells as well as the epithelial lining cells of the gastric glands and the epithelial cells of the serosa (Figures 4A-B). EMA reactivity was also noticed within the mucosal epithelial cells, and the basal cell membranes of most of the epithelial lining cells of the gastric glands (Figures 4B).

B- Indomethacin treated group

The microscopic examination of tissues of the stomach body after indomethacin administration showed moderate EMA reactivity of some luminal mucosal epithelial cells and the epithelial lining cells of the gastric glands (Figures 4C-D). Weak or negative EMA reactivity was displayed by some luminal mucosal epithelial cells (Figure 4D).

C- Melittin treated group

The stomach body tissues of mice treated with 10 µg/kg melittin for 3 days showed the extent of intense EMA reactivity at the luminal membranes of the mucosal epithelium (Figures 5A-B) as well as inside some of these cells (Figure 5B). Intense EMA reactivity was also displayed by the basal cell membranes of most of the epithelial cells of the gastric glands as well as at the epithelial cells of the serosa (Figures 5A-B). Similar EMA reactivity were displayed by the epithelial cells of the mucosal surface, the gastric glands and the serosa in the stomach body tissues of mice treated with the same dose of melittin for 5 days (Figures 5C-D) or 10 days (Figures 5E-F).

After treated with 40 µg/kg melittin, the stomach body tissues also showed intense EMA reactivity along the whole luminal surfaces of the mucosal epithelium as well as within some cells. Intense EMA reactivity were also noticed at the basal membranes of a large number of epithelial lining cells of the gastric glands as well as all the epithelial cells of the serosa when treated for 3 days (Figures 6A-B) or 5 days (Figures 6C-D), or 10 days of 40 µg/kg melittin (Figures 6E-F).

D- Indomethacin – Melittin treated group

The stomach body tissues from mice treated with indomethacin followed by treatment with melittin 10 µg/kg for 3 days showed negative to weak EMA reactivity at the luminal cell membranes of the mucosal epithelial cells, while the epithelial lining cells of the gastric glands revealed moderate reactivity. The epithelial cells of the serosa demonstrated intense EMA reactivity (Figures 7A-B). On the other hand, the body of the stomach of mice treated with indomethacin followed by treatment with 10 µg/kg melittin for 5 days showed moderate EMA reactivity of the mucosal epithelium and the epithelial lining cells of the gastric glands. However, some mucosal epithelial cells revealed negative EMA reactivity. The epithelial cells of the serosa showed intense EMA reactivity as usual (Figures 7C-D). Treatment with the same dose of melittin for 10 days to mice pretreated with indomethacin revealed the extent of intense EMA reactivity at the luminal cell membranes of the mucosal epithelium and at the basal cell membranes of many epithelial lining cells of the gastric glands (Figures 7E-F).

The body of the stomach of mice treated with indomethacin followed by treatment with 40 µg/kg melittin for 3 days showed moderate EMA reactivity at the luminal cell membranes of some mucosal epithelial cells; however the membrane of other cells showed weak or negative EMA reactivity (Figures 8A-B). Nevertheless, the body of the stomach of mice treated with indomethacin followed by treatment with 40 µg/kg melittin for 5 days showed the common intense EMA reactivity as in the control group, at the luminal cell membranes of the mucosal epithelium, within some of the mucosal epithelial cells and at the basal cell membranes of the epithelial lining cells of the gastric glands (Figures 8C-D). Moreover, the common extent of intense EMA reactivity at the luminal cell membranes of the mucosal epithelium and the basal cell membranes of many epithelial lining cells of the gastric glands was exhibited by the stomach body tissues of mice treated with 40 µg/kg melittin for 10 days (Figures 8E-F) in a similar manner to the EMA reactivity of the control sections.

Table 1. Histopathological observations in tissues of the body of the stomach in control and experimental groups

Groups / Observations	Control Group	Indometh. Group	Melittin Groups	Indomethacin-Melittin Group					
				10 µg/kg			40 µg/kg		
				3D	5D	10D	3D	5D	10D
Erosion	Absent	In some epithelial cells	Absent	Small foci	At a few points	Absent	Absent	Absent	Absent
Exfoliation	Absent	In some epithelial cells	Absent	Few	Absent	Absent	Absent	Absent	Absent
Inflammation	Absent	Large areas full of inflammatory cells at the submucosa and extend to the lamina properia	Absent	Few inflammatory cells at the submucosa	Less number of inflammatory cells	Absent	Noticeable reduction in number of inflammatory cells in the submucosa	Obvious decrease in number of inflammatory cells	Absent

Table 2. Immunohistochemical reactivity of the epithelial membrane antigen (EMA) at tissues of the stomach body

Group	Mucosal epithelial cells	Epithelial lining cells of gastric glands	Epithelial lining cells of the serosa
Control group	+ + +	+ + +	+ + +
Indomethacin group	+ ±	+ +	+ +
Melittin group (10 µg/kg) for 3 days	+ + +	+ + +	+ + +
Melittin group (10 µg/kg) for 5 days	+ + +	+ + +	+ + +
Melittin group (10 µg/kg) for 10 days	+ + +	+ + +	+ + +
Melittin group (40 µg/kg) for 3 days	+ + +	+ + +	+ + +
Melittin group (40 µg/kg) for 5 days	+ + +	+ + +	+ + +
Melittin group (40 µg/kg) for 10 days	+ + +	+ + +	+ + +
Indomethacin - Melittin group (10 µg/kg) for 3 days	±+	++	+++
Indomethacin - Melittin group (10 µg/kg) for 5 days	+ ±	+ +	++ +
Indomethacin - Melittin group (10 µg/kg) for 10 days	+ + +	+ + +	+ + +
Indomethacin - Melittin group (40 µg/kg) for 3 days	+±	++	++
Indomethacin - Melittin group (40 µg/kg) for 5 days	+ + +	+ + +	+ + +
Indomethacin - Melittin group (40 µg/kg) for 10 days	+ + +	+ + +	+ + +

(±) Negative to weak reactivity (+) Weak reactivity (+ ±) Weak to moderate reactivity (++) Moderate reactivity (+++) Intense reactivity

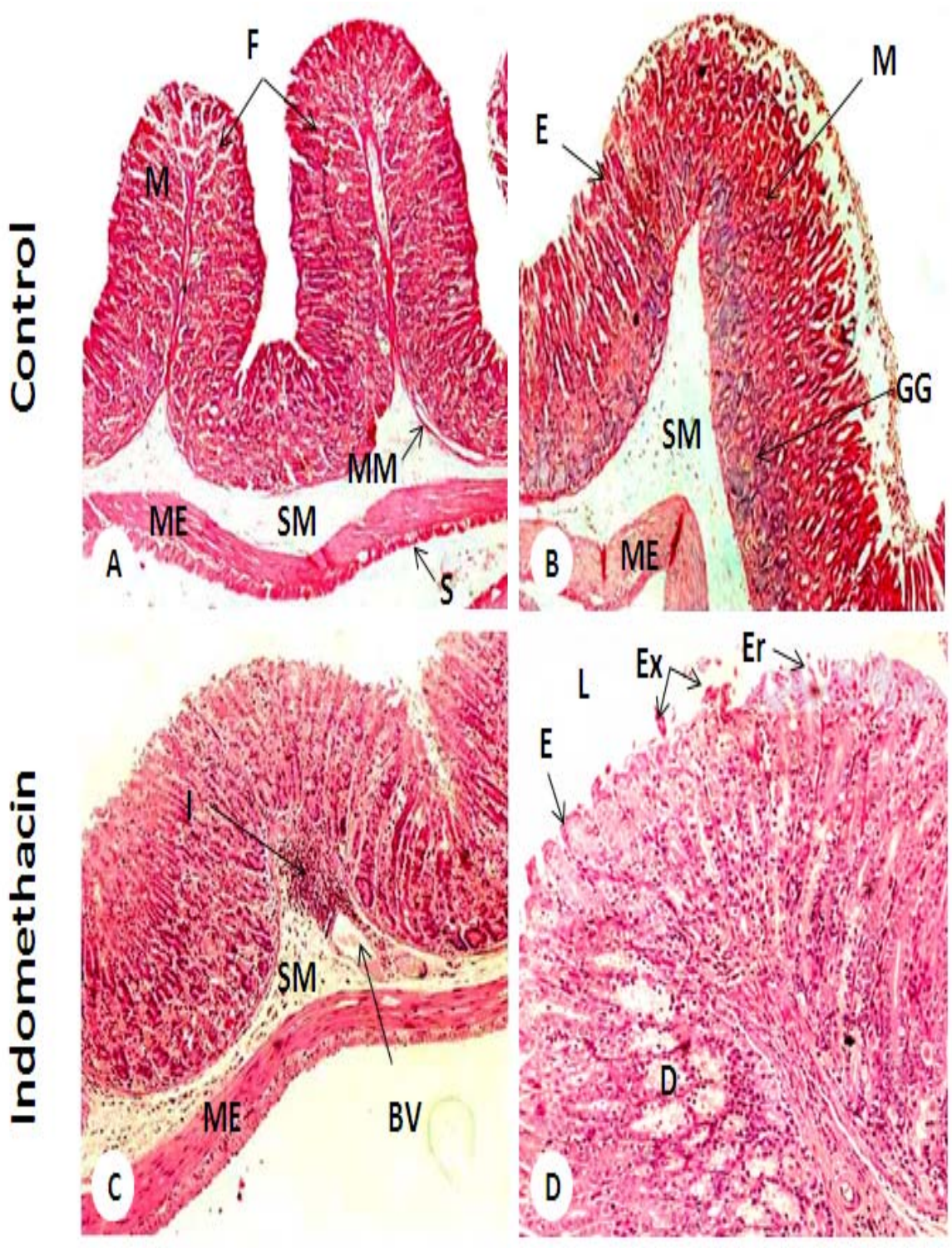

Figure 1. Effect of indomethacin on stomach body tissues

Transversesections in the body of the stomach of **(A)** a control mouse showing the four characteristic layers, the mucosa (M), the submucosa (SM), the muscularisexterna (ME) and the serosa (S). F: Mucosal folds; MM: Mascularis Mucosa (H&E, X100); **(B)** a control mouse showing enlarged mucosal fold that shows the mucosal epithelium (E) and the gastric glands (GG). M: Mucosa; SM: Submucosa; ME: Muscularisexterna (H&E, X100); **(C)** a mouse treated with indomethacin showing submucosal infiltration (I). SM: Submucosa, BV: blood vessel; ME: MuscularisExterna (H&E, X100); **(D)** a mouse treated with indomethacin showing enlarged part of a mucosal fold that displays erosion (Er) and exfoliation of some epithelial cells (Ex) to the gastric lumen (L). Note gastric glands with damaged lining epithelial cells (D). E: Intact epithelial cells (H&E, X400).

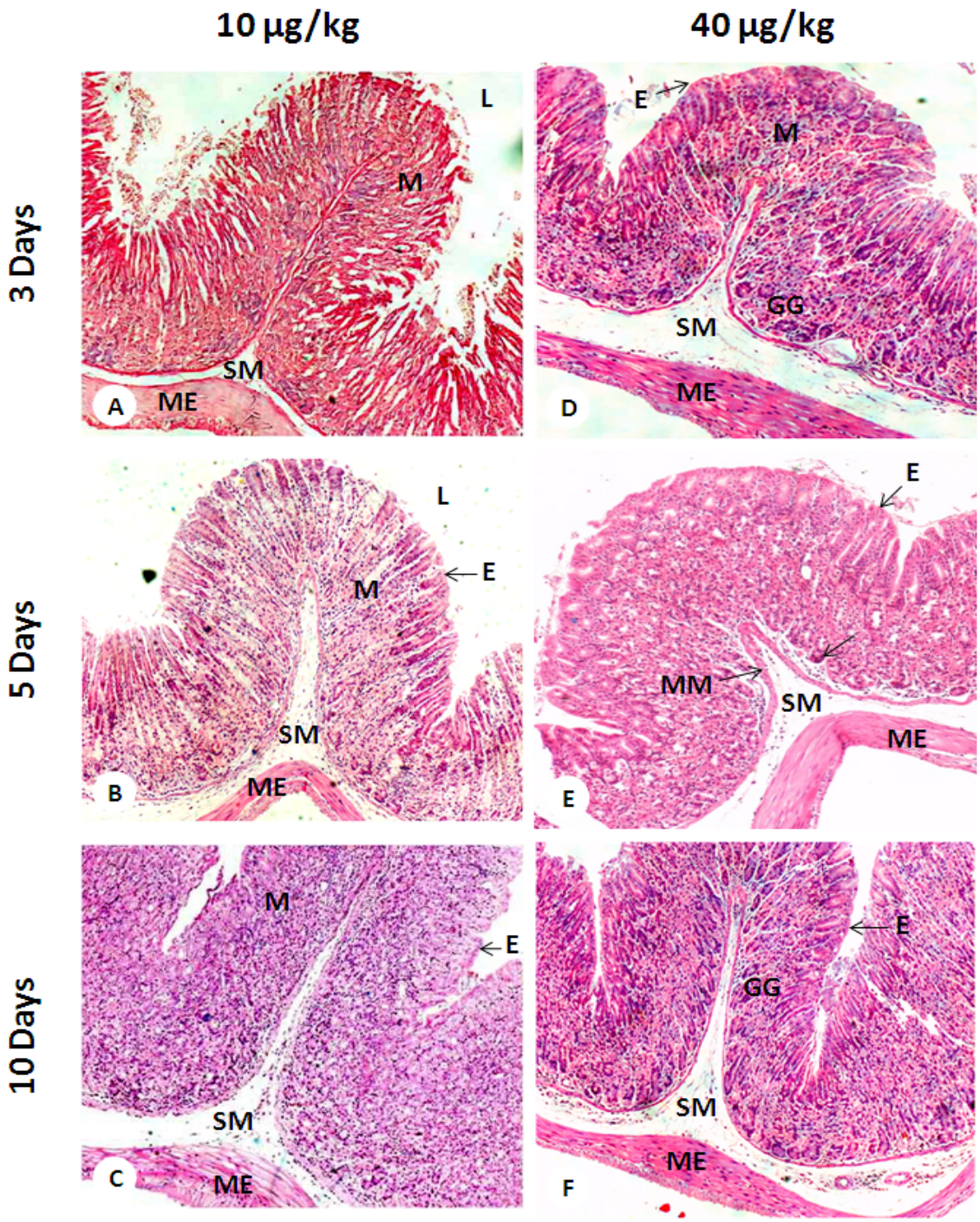

Figure 2. Effect of melittin on stomach body tissues

Transversesection in the body of the stomach of a mouse treated with melittin (10 µg/kg) **(A)** for 3 days showing the common characteristics of the stomach layers. L: Lumen; M: Mucosa; SM: Submucosa; ME: Muscularisexterna (H&E, X100); **(B)** for 5 days showing the normal histological features of the stomach layers. Note intact epithelium (E). L: Lumen; M: Mucosa; SM: Submucosa; ME: Muscularisexterna (H&E, X100); **(C)** for 10 days showing the normal histological pattern of the stomach layers with intact epithelium (E). M: Mucosa; SM: Submucosa; ME: Muscularisexterna (H&E, X100); **(D)** a mouse treated with melittin (40 µg/kg) for 3 days showing a mucosal fold that exhibits normal appearance of the gastric mucosa (GM) with intact mucosal epithelium (E) and normal histological structure of the gastric glands (GG) together with the common features of the submucosa (SM) and the muscularisexterna (ME) (H&E, X100); **(E)** for 5 days showing intact mucosal epithelial cells (E) together with the normal characteristics of the gastric glands (GG), the muscularis mucosa (MM), the submucosa (SM) and the muscularisexterna (ME) (H&E, X100); **(F)** for 10 days showing the mucosal fold that display their normal histological features. Note intact mucosal epithelium and normal appearance of gastric glands (GG). SM: Submucosa; ME: MuscularisExterna (H&E, X100).

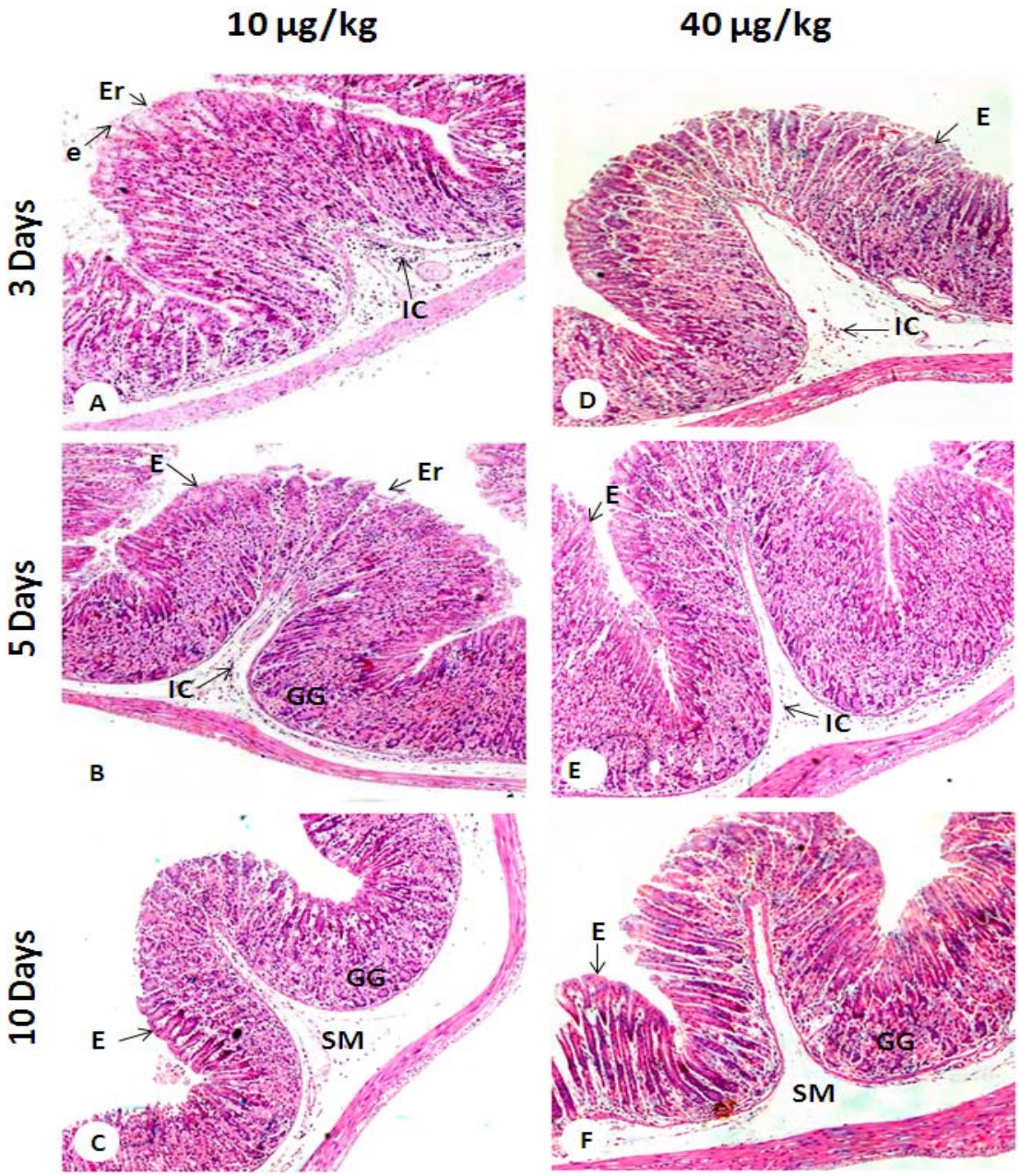

Figure 3. Protective effect of melittin (10 or 40 µg/kg) against stomach body inflammation

Transversesections in the body of the stomach of a mouse treated with indomethacin followed by treatment with melittin**(A)** (10 µg/kg) for 3 days showing fewer number of inflammatory cells (IC) compared to those observed in the indomethacin treated sections. Note the presence of a few exfoliated mucosal epithelial cells (e) and a locus of erosion (Er); **(B)** (10 µg/kg) for 5 days showing more or less intact epithelial mucosal cells (E) except for the presence of erosion (Er) at certain points. Note a few submucosal inflammatory cells (IC), but normal histological structure of the gastric glands (GG); **(C)** (10 µg/kg) for 10 days showing the normal appearance of the epithelial mucosal cells (E) and disappearance of most of the inflammatory cells, but the presence of a very few lymphocytes in the submucosa (SM). Note normal appearance of gastric glands (GG); **(D)** (40 µg/kg) for 3 days showing the reduced number of inflammatory cells (IC), besides the absence of erosion and the presence of mostly intact mucosal epithelial cells (E); **(E)** (40 µg/kg) for 5 days showing the common feature of the stomach layer with an obvious decrease in the occurrence of inflammatory cells (IC). Note intact mucosal epithelium (E); **(F)** (40 µg/kg) for 10 days showing the normal histological structure of the four gastric layers with complete disappearance of the inflammatory cells at the submucosa (SM) and the occurrence of intact mucosal epithelium (E) and gastric glands (GG) (H&E, X100).

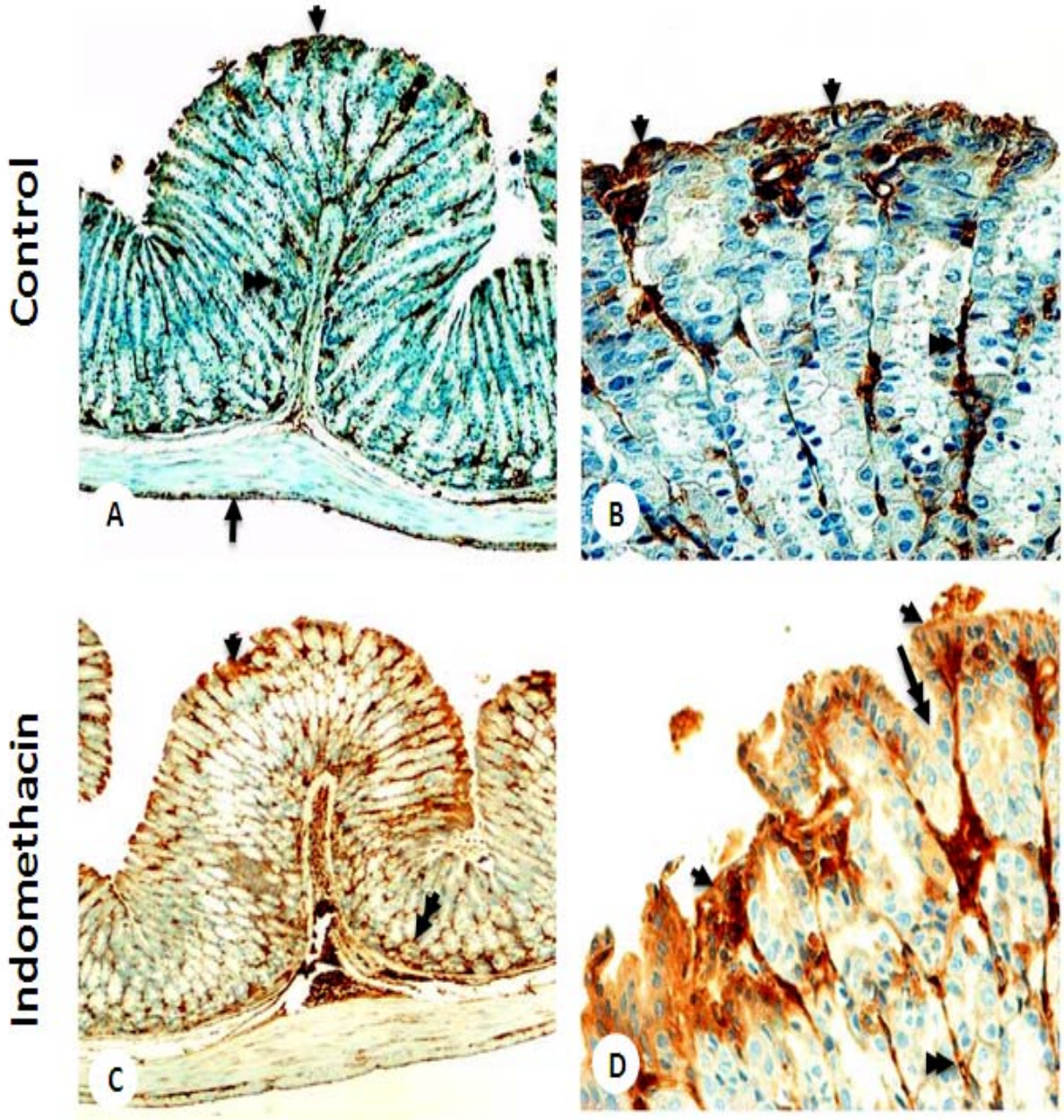

Figure 4. Effect of indomethacin on EMA reactivity

Transversesection in the body of the stomach of **(A)** a control mouse showing intense EMA reactivity at the mucosal epithelial cells (arrow head), the epithelial lining cells of the gastric glands (double arrow heads) and the epithelial cells of the serosa (arrow) (EMA immunohistochemistry, X100); **(B)** a control mouse showing intense EMA reactivity along the luminal (apical) cell membranes and within of the mucosal epithelial cells as well as within the cells (arrow heads), and the basal cell membranes of most of the epithelial lining cells of the gastric glands (double arrow heads) (EMA immunohistochemistry, X400), **(C)** the stomach of a mouse treated with indomethacin showing moderate EMA reactivity of some luminal mucosal epithelial cells (arrow head) and the epithelial lining cells of the gastric glands (double arrow heads) (EMA immunohistochemistry, X100); **(D)** a mouse treated with indomethacin showing enlarged part of the previous figure which display moderate EMA reactivity of the luminal mucosal epithelial cells (arrow heads) and epithelial cells of the gastric glands (double arrow heads). Note weak or negative EMA reactivity at some luminal mucosal epithelial cells (double arrows) (EMA immunohistochemistry, X400).

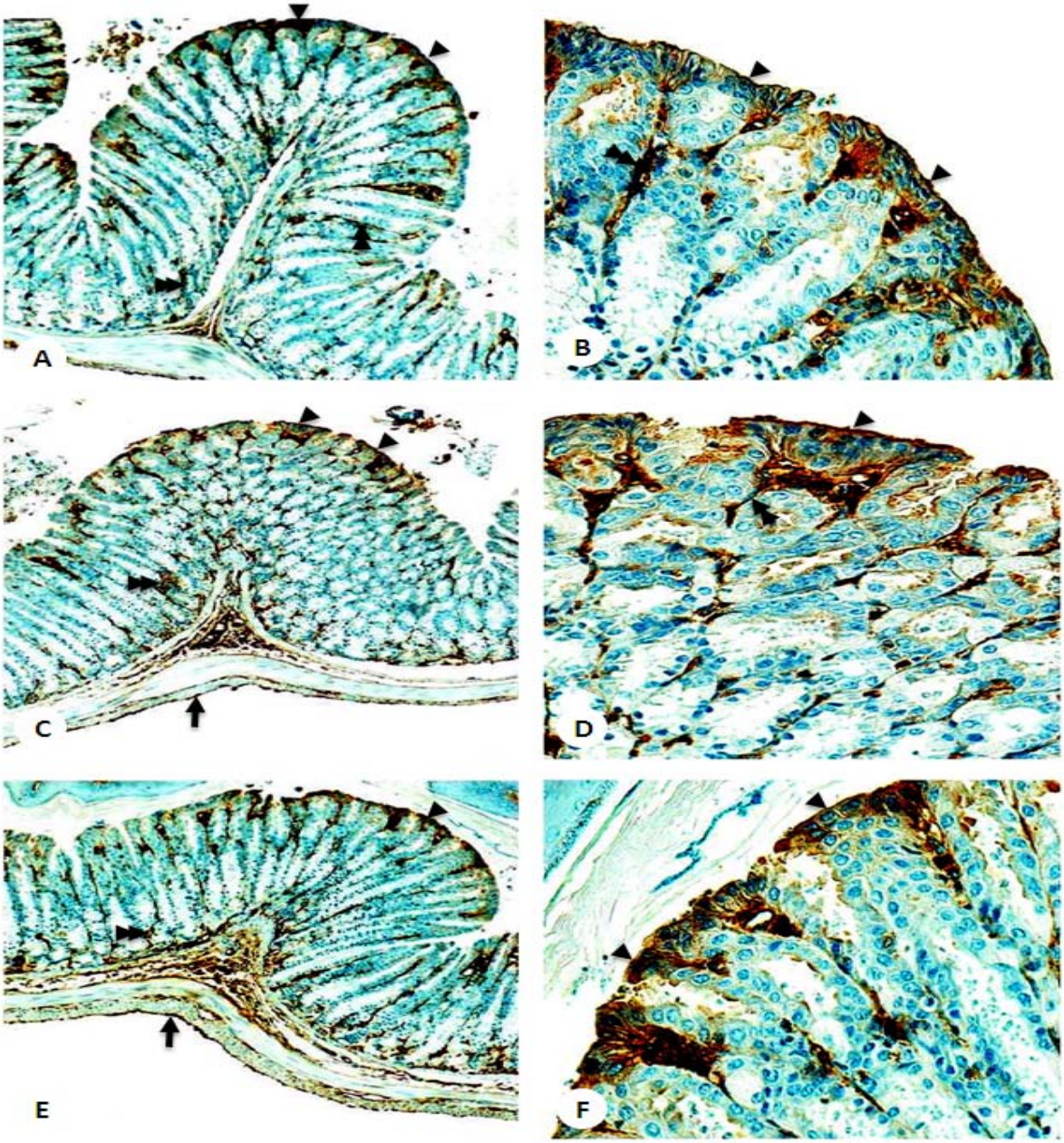

Figure 5. Effect of melittin(10 µg/kg) on EMA reactivity

(A-B) Transversesection in the body of the stomach of a mouse treated with melittin (10 µg/kg) for 3 days **(A)** showing intensely reacted mucosal epithelium (arrow heads) and the epithelial lining cells of the gastric glands (double arrow heads) (EMA immunohistochemistry, X100); **(B)** showing the extend of intense EMA reactivity at the luminal membranes of the mucosal epithelium (arrow heads) as well as within the cells. Note intense reactivity at the basal cell membranes of most of the epithelial cells of the gastric glands (EMA immunohistochemistry, X400).

(C-D) Transversesection in the body of the stomach of a mouse treated with melittin (10 µg/kg) for 5 days **(C)** showing intense reactivity with epithelium membrane antigen at the mucosal epithelium (arrow heads), the epithelial lining cells of the gastric glands (double arrow heads) and the serosa (arrow) (EMA immunohistochemistry, X100); **(D)** showing enlarged part of the previous figure in which the mucosal epithelium (arrow head) and the epithelial lining cells of the gastric glands (double arrow heads) reveal intense EMA reactivity (EMA immunohistochemistry, X400).

(E-F) Transversesection in the body of the stomach of a mouse treated with melittin (10 µg/kg) for 10 days **(E)** showing the luminal mucosal epithelium (arrow head), the epithelial lining cells of the gastric glands (double arrow heads) and the epithelial cells of the serosa (arrow) that intensely reacted with epithelium membrane antigen (EMA immunohistochemistry, X100); **(F)** Transversesection in the body of the stomach of a mouse treated with melittin (10 µg/kg) for 10 days showing enlarged part of the mucosal epithelium (arrow heads) that intensely reacted with EMA (EMA immunohistochemistry, X400).

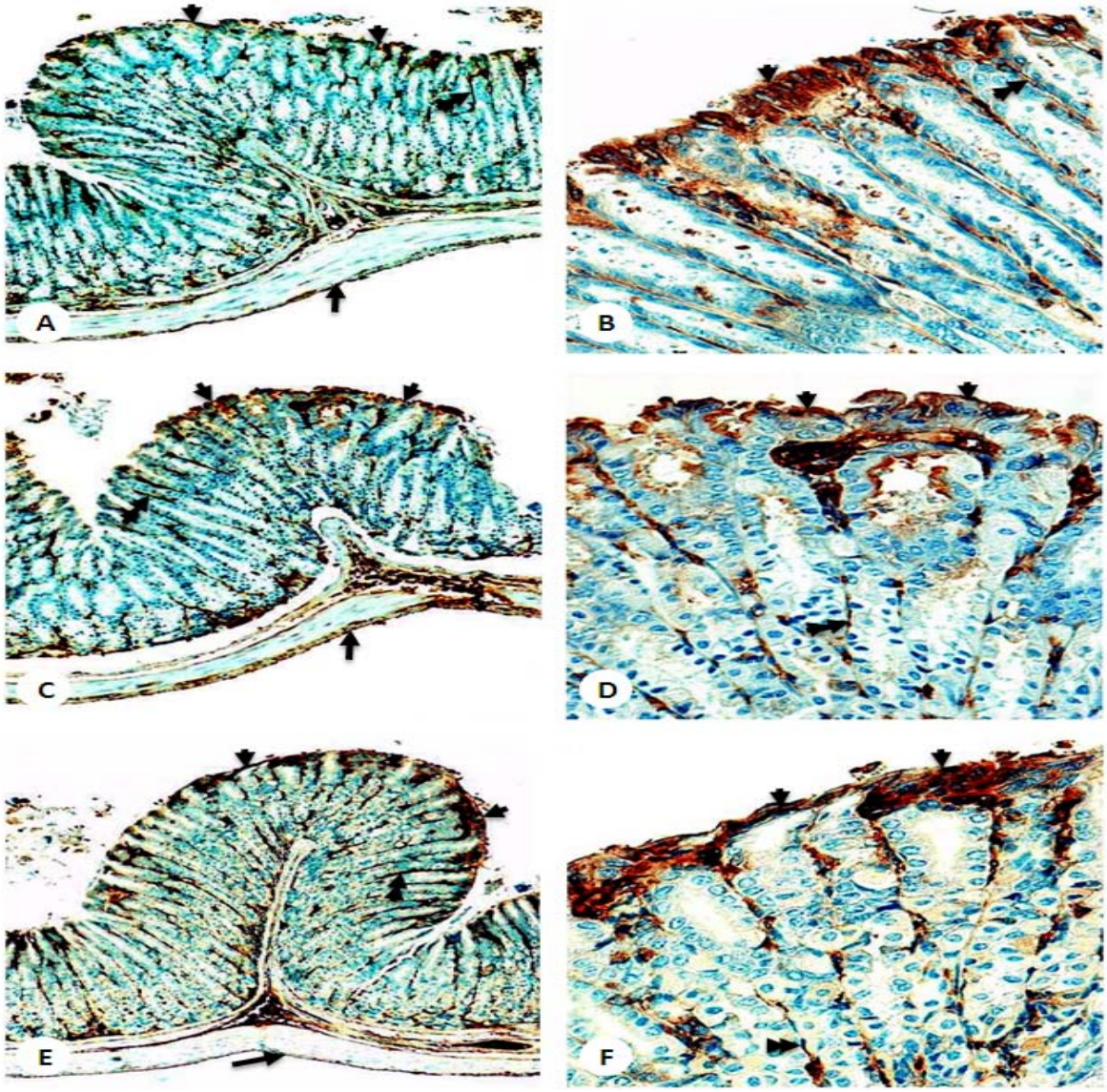

Figure 6. Effect of melittin(40 µg/kg) on EMA reactivity

(A-B) Transversesection in the body of the stomach of a mouse treated with melittin (40 µg/kg) for 3 days **(A)** showing intense reactivity with EMA at the luminal cell membranes of the mucosal epithelium (arrow heads) and the basal membranes of many epithelial lining cells of the gastric glands (double arrow heads). Arrow indicate intense EMA reactivity of the epithelial cells of the serosa (EMA immunohistochemistry, X100); **(B)** showing enlarged part of the mucosal epithelium (arrow head) displaying intense reactivity with EMA. Note intense reactivity at the basal cell membranes of many epithelial lining cells of the gastric glands (EMA immunohistochemistry, X400).

(C-D) Transversesection in the body of the stomach of a mouse treated with melittin (40 µg/kg) for 5 days **(C)** showing intense EMA reactivity at the mucosal epithelium (arrow heads), the epithelial lining cells of the gastric glands (double arrow heads) and the serosal epithelial cells (arrow) (EMA immunohistochemistry, X100); **(D)** showing intense EMA reactivity at the luminal cell membranes of the mucosal epithelium (arrow heads) and within some cells. Note intense reactivity of the basal membranes of some epithelial lining cells of the gastric glands (double arrow heads) (EMA immunohistochemistry, X400).

(E-F) Transversesection in the body of the stomach of a mouse treated with melittin(40 µg/kg) for 10 days **(E)** showing intense EMA reactivity along the whole luminal surfaces of the mucosal epithelium (arrow heads) as well as within some cells. Intense reactivity can be seen at many epithelial lining cells of the gastric glands (double arrow heads) and the epithelial cells of the serosa (arrow) (EMA immunohistochemistry, X100); **(F)** showing enlarged part of the previous figure which reveal intense reactivity at the luminal cell membranes of the mucosal epithelium (arrow heads) and within some cells. The basal cell membranes of the epithelial lining cells of the gastric glands reveal intense reactivity (double arrow heads) (EMA immunohistochemistry, X400).

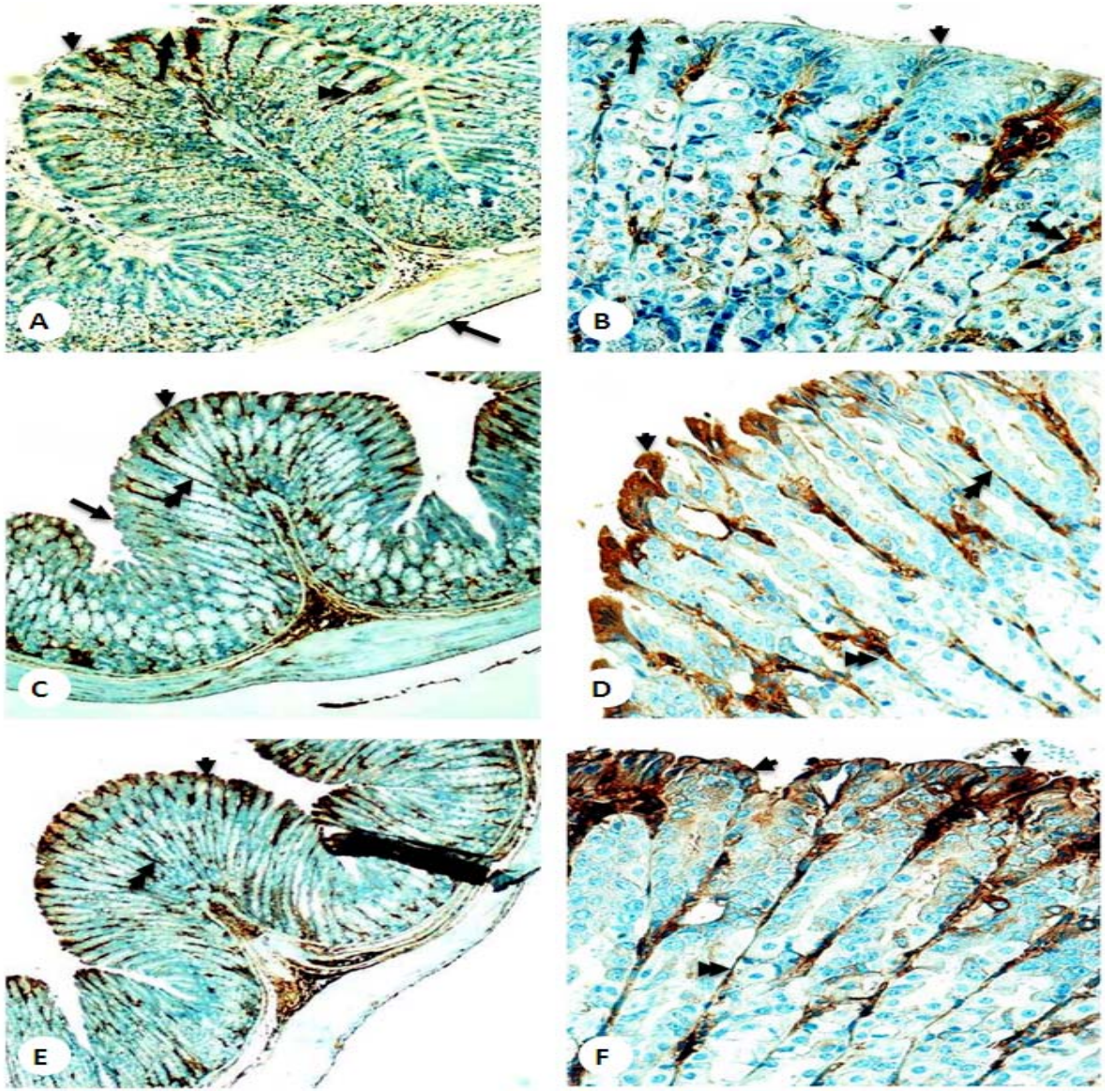

Figure 7. Protective effect of melittin(10 µg/kg) against stomach body inflammation

(A-B) Transversesection in the body of the stomach of a mouse treated with indomethacin followed by treatment with melittin (10 µg/kg) for 3 days **(A)** showing negative (double arrow) to weak (arrow head) EMA reactivity at the luminal cell membranes of the mucosal epithelial cells (arrow head). The epithelial lining cells of the gastric glands (double arrow heads) reveal moderate reactivity. Arrow indicates intense reactivity at the epithelial cells of the serosa (EMA immunohistochemistry, X100); **(B)** showing the mucosal epithelium with weak (arrow head) or negative (double arrows) EMA reactivity. Note moderate EMA reactivity of the basal membranes of some epithelilal lining cells of the gastric glands (double arrow heads) (EMA immunohistochemistry, X400).

(C-D) Transversesection in the body of the stomach of a mouse treated with indomethacin followed by treatment with melittin (10 µg/kg) for 5 days **(C)** showing moderate EMA reactivity of the mucosal epithelium (arrow head) and the epithelial lining cells of the gastric glands (double arrow heads). Note negative EMA reactivity of some mucosal epithelial cells (arrow) (EMA immunohistochemistry, X100); **(D)** showing enlarged part of the previous figure which indicate moderately reacted mucosal epithelium (arrow head) and the basal cell membranes of the epithelial lining cells of the gastric glands (double arrow heads) (EMA immunohistochemistry, X400).

(E-F) Transversesection in the body of the stomach of a mouse treated with indomethacin followed by treatment with melittin (10 µg/kg) for 10 days **(E)** showing the extend of intense EMA reactivity at the luminal cell membranes of the mucosal epithelium (arrow head) and at the basal cell membranes of many epithelial lining cells of the gastric glands (double arrow heads) (EMA immunohistochemistry, X100); **(F)** showing intense reactivity with EMA at the luminal cell membranes of the mucosal epithelium (arrow head) and within some cells. The basal cell membranes of many epithelial lining cells of the gastric glands (double arrow heads) reveal intense reactivity (EMA immunohistochemistry, X400).

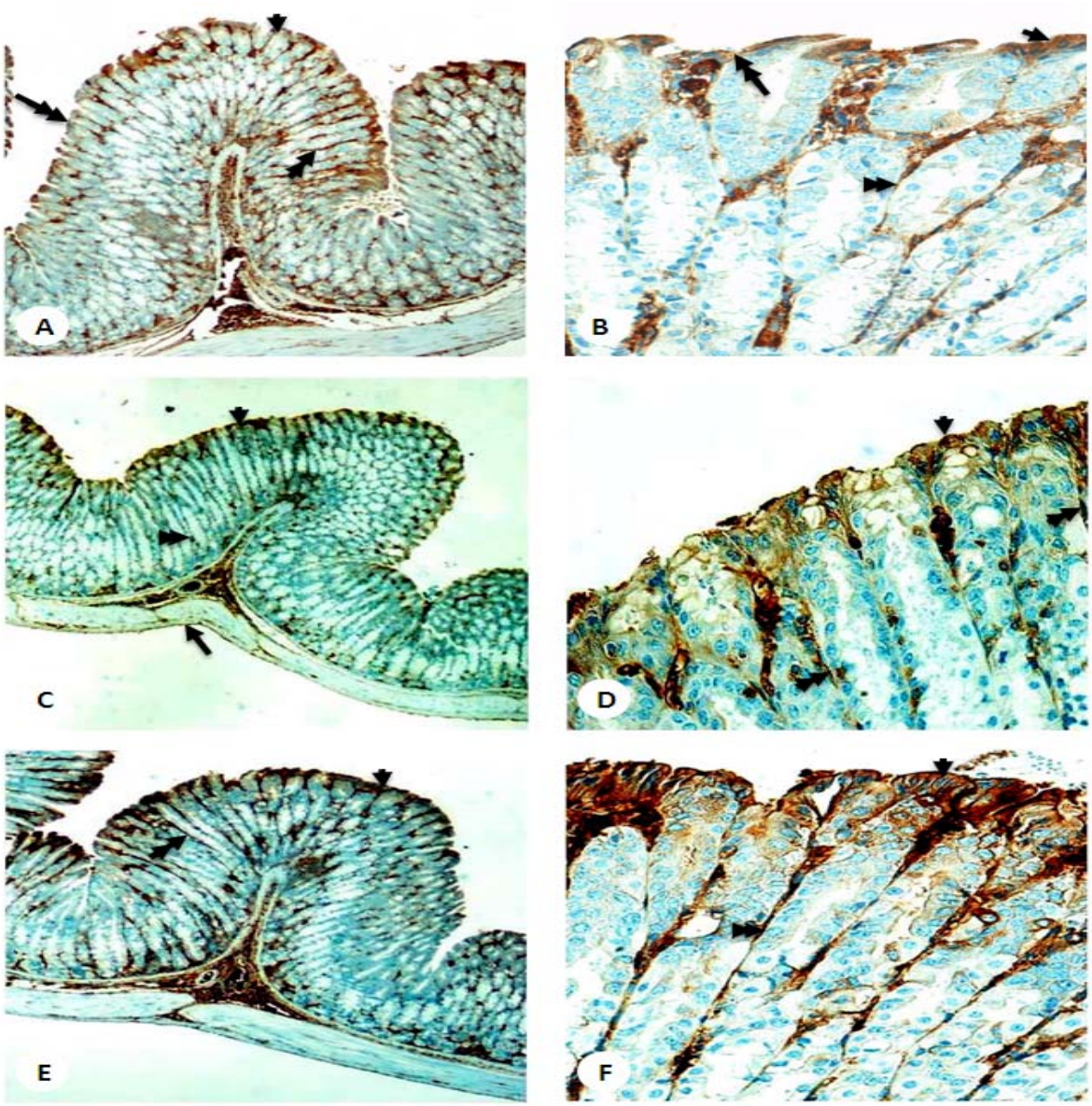

Figure 8. Protective effect of melittin(40 µg/kg) against stomach body inflammation

(A-B) Transversesection in the body of the stomach of a mouse treated with indomethacin followed by treatment with melittin (40 µg/kg) for 3 days **(A)** showing moderate EMA reactivity at the mucosal epithelium (arrow head) and the epithelial lining cells of the gastric glands (double arrow heads). Double arrows indicate weak EMA reactivity of some mucosal epithelium (EMA immunohistochemistry, X100); **(B)** showing enlarged part of previous figure that display moderately reacted mucosal epithelium (arrow head) and epithelial lining cells of the gastric glands (double arrow heads) with EMA. Note weak EMA reactivity of some mucosal epithelium (double arrows) (EMA immunohistochemistry, X400).

(C-D) Transversesection in the body of the stomach of a mouse treated with indomethacin followed by treatment with melittin (40 µg/kg) for 5 days **(C)** showing the intense EMA reactivity at the luminal cell membranes of the mucosal epithelium (arrow head) and the basal cell membranes of the epithelial lining cells of the gastric glands (double arrow heads). Arrow indicate intense reactivity at the epithelial cells of the serosa (EMA immunohistochemistry, X100); **(D)** showing intensely reacted mucosal epithelium (arrow head) and the epithelial lining cells of the gastric mucosa (double arrow heads) with EMA (EMA immunohistochemistry, X400).

(E-F) Transversesection in the body of the stomach of a mouse treated with indomethacin followed by treatment with melittin (40 µg/kg) for 10 days **(E)** showing the common extend of intense reactivity with EMA at the luminal cell membranes of the mucosal epithelium (arrow head) and the basal cell membranes of many epithelial lining cells of the gastric glands (double arrow heads) (EMA immunohistochemistry, X100); **(F)** showing the mucosal epithelium (arrow head) and the epithelial lining cells of the gastric glands (double arrow heads) which are intensely reacted with EMA in a similar manner to the EMA reactivity of the control sections (EMA immunohistochemistry, X400).

4.Discussion

The gastrointestinal (GI) tract is highly susceptible to inflammatory responses because of its enormous mucosal surface, which is continuously exposed to a myriad of antigenic, mitogenic, mutagenic and toxic stimuli (Fiocchi, 1997). The causes of inflammation in the GI tract are varied and include microbial infection,

chemical materials, enzymatic injury as well as autoimmune processes (Johnson, 2006). Indomethacin is the most widely used drugs for various acute or chronic conditions, such as relief of pain and inflammation, prevention of colorectal cancer (Baron *et al*., 2006), and treatment of cancer (Kerr *et al*., 2007) and cardiovascular disease (Berger *et al*., 2009). On the other hand, their use is strongly associated with a broad spectrum of unexpected adverse effects in various organs (Lanas andScarpignato, 2006). Particularly, GI inflammation is the most common adverse events of these agents (Serrano *et al*., 2002). In the present study, a single dose of indomethacin (50 mg/kg) was selected as the minimum dose that causes inflammation in the GI of the experimental animals. The results of the histological studies showed that the effect of indomethacin on the stomach body tissue of mice included erosion and exfoliation of some mucosal epithelial cells besides the occurrence of large foci of numerous inflammatory cells at the submucosa. Similar observations were reported by Nakajima *et al*. (2012) who demonstrated that indomethacin produced mucosal damage, infiltration and activation of inflammatory cells as a side effect. In the stomach, prostaglandins (PG), especially PGE2, play an important role in maintaining gastric mucosal integrity via several mechanisms, including the regulation of gastric mucosal blood flow, turnover of epithelial cells, synthesis of mucus, and inhibition of gastric acid secretion (Wallace, 1992). Previous studies on the mice stomach showed that the gastric toxicity of NSAIDs compounds like indomethacin can suppress the production of PGs by inhibiting cyclo-oxygenase (COX) enzymes; COX-1 and COX-2 (Adhikary*et al*., 2012). This result suggested that the inhibition of PGs by indomethacin, in turn, can lead to gastric inflammation and ulceration as a side effect.

On the other hand, the microscopic examination of H&E stained sectioned of stomach healthy mice were orally treated either by 10 or 40 µg/kg melittin for 3, 5 or 10 days showed the normal appearance of the stomach four layers(Yun *et al*., 2011). In the present study, both doses of melittin (10 and 40 µg/kg) showed no structuralchanges of the stomach tissues.

The uses of melittin for treatment of inflammation in different tissues were reported by several investigators, such as the protective effect of melittin on inflammation induced in case of osteoarthritic chondrocytes (Nah *et al*., 2007), rheumatoid arthritis (Stuhlmeier, 2007; Li *et al*., 2010) acute pancreatitis (Yun *et al*., 2011) and acute liver inflammation (Park *et al*., 2012). However, on reviewing the available literature it was found that no studies were published concerning the effect of melittin on inflammation induced in the GI. The results of the present study indicated that treatment with melittin can reduce inflammation in stomach tissues in a dose and time dependent manner. Complete disappearance of inflammation was achieved in tissues of mice treated for 10 days with any of the used doses (10 or 40 µg/kg melittin). The anti-inflammatory effect of melittin may be attributed to multiple inhibitory effect on the generation of the inflammation mediators such as nitric oxide (NO) and tumor necrosis factor (TNF-α) as well as on the release of intracellular calcium (Lee *et al*., 2004).

The present study also showed the disappearance of erosion that induced by indomethacin treatment, after 10 days in stomach of treatment with 10 µg/kg melittin as well as after 3 days of treatment with 40 µg/kg of melittin to indomethacin-treated mice. Nevertheless, exfoliations disappeared from stomach tissues due to treatment of indomethacin-treated mice with any dose of melittin for any duration except in indomethacin-treated mice that treated with 10 µg/kg melittin for 3 days, which showed few exfoliations. It is believed that the disappearance of erosion and exfoliation in these tissues could be attributed to the ability of melittin to protect the epithelial lining cells of the stomach. This suggestion can be confirmed by the results of Lee *et al*. (2011) and Park *et al*. (2012) that invistgated the effect of melittin on the liver epithelial cells during inflammation, and suggested that the low doses of melttin significantly protected liver epithelial cells from damage through the inhibition of inflammatory cytokines and apoptosis. In addition, Yun *et al*. (2011) mentioned that melittin reduced serum cytokines (IL-1B, IL-6, and TNF-α), which contribute at an early stage to damage of cells during jun N-terminal kinases (JNK) pathway, so necrosis and damage occurred in the pancreas. The role of melittin in reducing the erosion and exfoliations may also be due to indirect action by suppressing the activity of phospholipase and protease enzymes. In this concern, Suh*et al*. (2006) demonstrated that low doses of melittin suppressed the activity of protease enzymes that may contribute to the exfoliation and erosion of the mucosal epithelial cell. Moreover Saini*et al*. (1997) reported that melittin bind to the phospholipase enzyme and inhibiting its enzymatic activity on the phospholipids of the cell membranes, which may contribute to maintain the integrity of these membranes and consequently reduce cellular damage in the mucosal epithelia. In contrast, high doses of melittin caused disruption of the cell structure and a necrotic loss of plasma membrane integrity by attacking all lipid membranes, leading to significant toxicity, thereby precluding any meaningful therapeutic benefit (Hoskin and Ramamoorthy, 2008; Hoshino *et al*., 2012).

In the present study, the immunohistochemical techniques were used to determine the Epithelial Membrane Antigen (EMA) that may reflect the integrity and the sensitivity of epithelial cells in the stomach tissues of indomethacin and/or melittin treated mice. The loss of the reactivity of such antigen in stomach tissues of mice treated with indomethacin could confirm the damaging effect of such agent on the mucosal epithelial cell the recorded in the regular histological studies. Several investigators mentioned that the severe gastrointestinal side effects of NSAIDs (including indomethacin) have to some extent been attributed to the inhibition of the prostaglandin-synthesizing enzymes which are important in gastric and intestinal mucosal protection with concomitant diminish of the gastric EMA (Hawkey, 2000; Holm, *et al*., 2002). Nevertheless, restoring of the reactivity of EMA in tissues of mice treated with indomethacin followed by treatment with melittin confirmed the protective effects of melittin to ameliorate the apical cell membranes of the mucosal epithelial cells and to protect them from ulceration and exfoliation.

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