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Full Length Research Paper

Anti-inflammatory effect of Melittin on Mice Jejunum

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Melittin is a principle toxic peptide of bee venom. It is known as a strong anti-inflammatory agent and used as traditional medicine for treatment of different types of diseases. 5-HT contributes at early stages on inflammatory processes in response to the local inflammation. However, the anti-inflammatory effect of melittin on the gastrointestinal (GI) tract has not been elucidated. The aim of the present study was to investigate the physiological changes of melittin on mice jejunum treated with indomethacin to induce inflammation. This study was performed on adult Swiss male mice. These mice divided into 4 groups (7 mice for each group): Control group: mice treated with distilled water; Indomethacin group treated with indomethacin (50 mg/kg) for 1day; Melittin group treated daily with melittin (10 or 40 µg/kg) for 3, 5 and 10 days; Indomethacin-melittin group treated with indomethacin followed by the above melittin doses. Samples from the jejunum were collected and prepared for physiological studies. Physiological study showed a significant increase of pro-inflammatory mediator 5-HT in the mucosal tissues of inflamed jejunum compared to control (337 vs 150 pg/ml), while this level was gradually reduced by melittin treatment 10 µg/kg for 3, 5 and 10 days (202, 185 and 170 pg/ml, P<0.05 respectively) and by melittin treatment 40 µg/kg for 3, 5 and 10 days (188, 163 and 148 pg/ml P<0.05 respectively). Melittin attenuated the inflammation of jejunum by inhibiting the release of pro-inflammatory mediator (5-HT). These data supported the potential use of melittin as anti-inflammatory therapy of GI inflammation.

Keywords: Melittin. Anti-inflammatory. Gastrointestinal.

INTRODUCTION

Honey bee venom (apitoxin) is synthesized in the venom glands of workers and queen bees from a mixture of acidic and basic secretions to form an acidic secretion with pH 4.5-5.5, and stored in their venom sacs. It is a complex mixture containing simple organic molecules, proteins, peptides, and other bioactive elements. Several of these components have been isolated and characterized, and their primary structures were determined by biochemical techniques (De Lima and Brochetto-Braga, 2003). Son *et al*. (2007) reported that bee venom contains a variety of peptides, including melittin, apamin, adolapin, the mast-cell-degranulating (MCD) peptide, enzymes (phospholipase A2), biologically active amines (histamine and epinephrine), and non-peptide components, which have a variety of pharmaceutical properties. Some of these components have been associated to allergic reactions, among several other symptoms.

Melittin is the principal toxic component in the venom of the honey bee, *Apis mellifera* (Hoskin and Ramamoorthy, 2008). It is a well-characterized pore-

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forming lytic amphiphilic peptide susceptible to be vehiculized in lipid membranes (Falco *et al*., 2013). This amphiphilic property of melittin allowed it to be used as a suitable model peptide for monitoring lipid-protein interactions in the membranes (Raghuraman and Chattopadhyay, 2007); however it is considered as a very nonspecific cytolytic peptide that attacks all lipid membranes (Hoskin and Ramamoorthy, 2008). The basis of melittin's action is a physical and chemical disruption of the membrane structure resulting in profound compromise of the cell permeability barrier (Yang *et al*., 2001).

Although there have been numerous studies on the effect of bee venom and melittin in the treatment of many diseases of various body organs, research concerning their role in the treatment of diseases of the digestive tract is still limited. Generally, bee venom and melittin are known to inhibit inflammatory reactions induced in various cell types (Park *et al*., 2011).

The therapeutic usage of melittin on liver had been reported by several investigators. Liu *et al*. (2008) mentioned that melittin have inhibitory effects on hepatocellular carcinoma HCC, where it can drastically inhibit cell motility and prevent HCC metastasis *in vivo*, suggesting that melittin is a potential therapeutic agent for HCC. Moreover, bee venom (Lee *et al*., 2011) and melittin (Park *et al*., 2011) could be considered as effective agents for preventing liver fibrosis, which is a process of healing and scarring in response to chronic liver injury, as they can attenuates liver injury in mice through modulating inflammation and fibrogenesis via suppressing the expression of pro-inflammatory cytokines through the nuclear factor (NF)-κB signaling pathway. Previous studies also referred to the ability of bee venom and melittin to treat pancreatitis. Seo *et al*. (2008) investigated the effect of bee venom on cholecystokinin octapeptide-induced acute pancreatitis in rats. They showed that bee venom reduced histological damages in pancreas.

Inflammation in any part of the gastrointestinal (GI) tract can profoundly influence the function of the mucosal layer that lies closest to the luminal contents (Wallace and Ma, 2001). The inflammatory response is coordinated, to a large extent, by an array of chemical mediators that are released from the epithelium and from the immunocytes and nerves within the lamina properia. Several components of the mucosal defense can be influenced by inflammatory agents such as serotonin (Wallace, 2001). Serotonin (5-Hydroxytryptamine) is a major transmitter molecule within the GI tract (Grundy, 2008). The biologic effects of serotonin in specific tissues are governed by the characteristics of the receptor to which serotonin attaches (Crowley, 2012).

NSAIDs are a large class of drugs which widely used for the treatment of pain, fever and inflammation (Hagos, 2008). Indomethacin is a common type of NSAIDs. With a continuous use for a long time or using a high dose, indomethacin cause numerous side effects including GI inflammation and ulceration and it caused significant injury to the stomach (Olaleye *et al*., 2013). The main mechanism of NSAID-induced GI injuries is inhibition of cyclo-oxygenase, mitochondrial dysfunction, oxidative stress, and enhancement of intestinal permeability (Bjarnason and Takeuchi, 2009; Fukumoto *et al*., 2011).

The present work aimed at studying the possibility of using melittin, one of the products of the bee venom, to ameliorate the physiological, histopathological and immunohistochemical alterations induced by oral administration of indomethacin, a nonsteroidal anti-inflammatory drug, on the stomach and jejunum of mice. The study is designed to point out to the risk of excessive use of indomethacin on the GI, and to demonstrate the safety of using melittin as an anti-inflammatory product of bee venom.

This study may help to support a potential strategy of using melittin for prevention gastrointestinal inflammation, where the effects of melittin on inflammation of the gastrointestinal tract have not been elucidated until now.

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

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.

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).

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).

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 24h from each treatment, mice of all groups were sacrificed under light ether anesthesia. Samples from the stomach body and jejunum collected from all animals were prepared for physiological study.

Physiological studies

Physiological studies were performed using Enzyme-Linked Immunosorbent Assay (ELISA) kits (obtained from USCNK company) to determine the release of pro-inflammatory agent (5-HT) 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*. (2001). The method included the following steps. Firstly, Jejunum segments were cut along the mesentery of each mouse. Then, the mucosa was removed by sharp dissection under a stereomicroscope on ice. After that, the dissected mucosa was snap-frozen in liquid nitrogen and stored at -70°C for later processing. Next, the tissue samples were gently blotted dry, weighed, and placed in ice-cold Tris-EDTA buffer (10 mM Tris HCl and 1 mM EDTA, pH 7.4) containing 0.05 g sodium azide, 1 ml Tween 80, 2 mM phenyl methyl sulfonyl fluoride, and 1 µg/ml of each of the protease inhibitors antipain, aprotinin, leupeptin, and pepstatin A at 100 mg of tissue per 1 ml of buffer. After that, the mucosal samples were placed on ice, minced, homogenized for 20 second and centrifuged at 11,000 *g* for 10 min at 4°C. Then, the supernatants were collected and filtered (0.45-mm Acrodisc). Finally, 5-HT levels were assayed by ELISA kits according to the manufacturer's instructions (USCNK Company).

Data analysis

The concentrations of the pro-inflammatory agent (5-HT) 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 $P < 0.05$.

RESULTS

5-HT Concentration of jejunum

Control group

The concentration of 5-HT in isolated mucosa of control mice jejunum was 150±4 pg/ml, (n=7). This value was compared with the level of 5-HT in experimental groups to study the effectiveness of melittin on inflammation.

Indomethacin treated group

Oral administration of indomethacin (50 mg/kg/1 day) significantly increased the level of 5-HT concentration in mucosal jejunum compared to control (337±5 *vs* 150±4 pg/ml, P<0.000, n=7, Figure 1, Table 1).

Melittin treated group

Effect of melittin (10 µg/kg)

The level of 5-HT in mice treated with 10 µg/kg of melittin

Figure 1

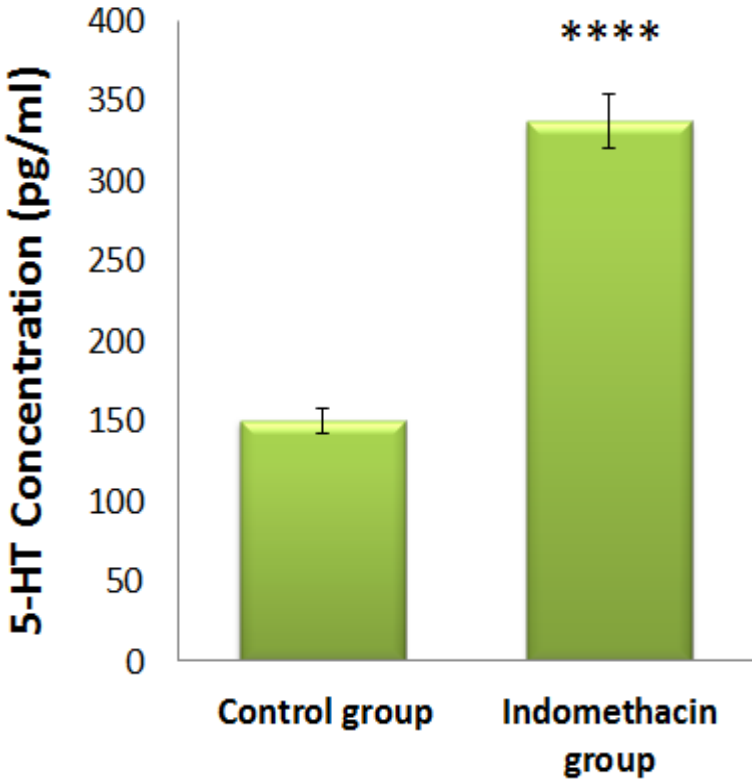

Figure 1. Histogram showing the concentration of 5-HT in indomethacin treated mice compared to control group. Note that the level of 5-HT in indomethacin group was a highly increased compared to control group. $^{****}P< 0.0001$.

Table 1. Mean values of 5-HT concentration ± SE in pg/ml in mucosal tissues of the jejunum in indomethacin treated mice compared to control group. $^{****}P< 0.0001$. Paired-samples *t*-test, *n*=7.

Groups	Control Group	Indomethacin Group
5-HT Con.	150 ± 4	337 ± 5****

for 3, 5 or 10 days was slightly reduced compared to control (138±4, 125±4 and 124±4 *vs* 150±4 pg/ml, *P*>0.8, 0.09, 0.06 respectively, *n*=7, Figure 2, Table 2).

Effect of melittin (40 µg/kg)

5-HT concentration in mice treated with 40 µg/kg for 3, 5 or 10 days was decreased significantly compared to control (118±4, 117 ± 4 and 113±4 *vs* 150±4 pg/ml, *P*<0.04, 0.02 and 0.03 respectively, *n*=7, Figure 3, Table 3).

Indomethacin-melittin treated group

Effect of melittin (10 µg/kg)

The mucosal 5-HT concentration in indomethacin-melittin (10 µg/kg) treated jejunum for 3, 5 or 10 days was diminished significantly compared to inflamed group (202±4 , 185±3 and 170±3 *vs* 337±5 pg/ml, *P* < 0.000, 0.002 and 0.001 respectively, *n*=7, Figure 4, Table 4).

Effect of melittin (40 µg/kg)

The level of 5-HT in indomethacin-melittin (40 µg/kg)

Figure 2

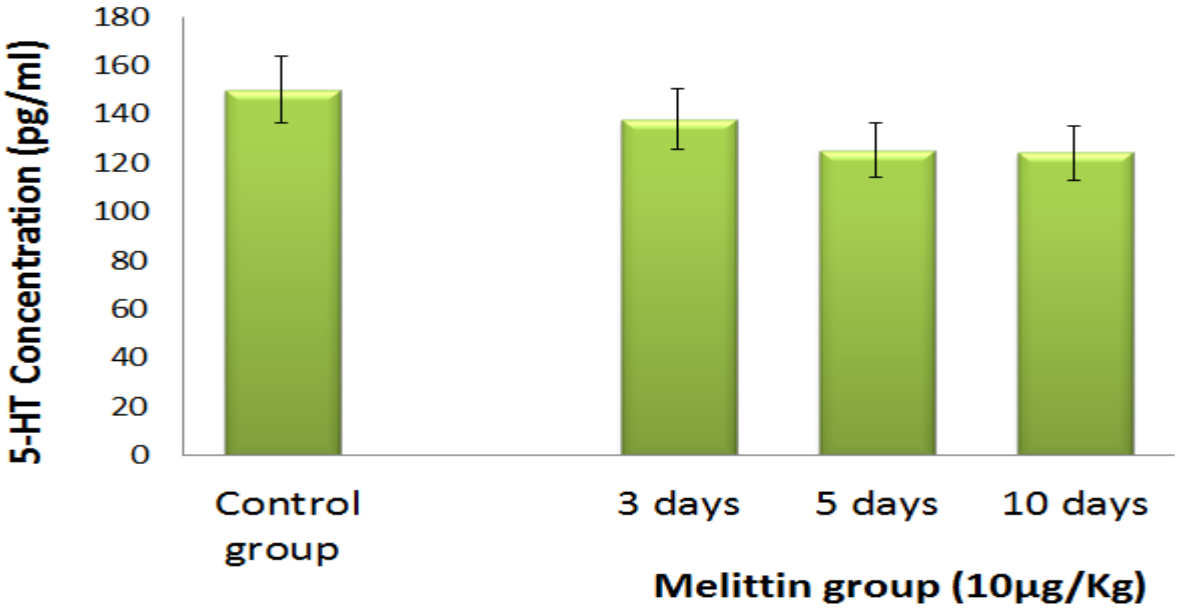

Figure 2. Histogram showing the concentration of 5-HT in melittin group (10 µg/kg) compared to control group. Note that 5-HT level in mice treated with 10 µg/kg of melittin for 3, 5 or 10 days was slightly reduced compared to control.

Table 2. Mean values of 5-HT 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.

Groups	Control Group	Melittin Group10 µg/kg		
		3 days	5 days	10 days
5-HT Con.	150 ± 4	138 ± 4	125 ± 4	124 ± 4

Figure 3

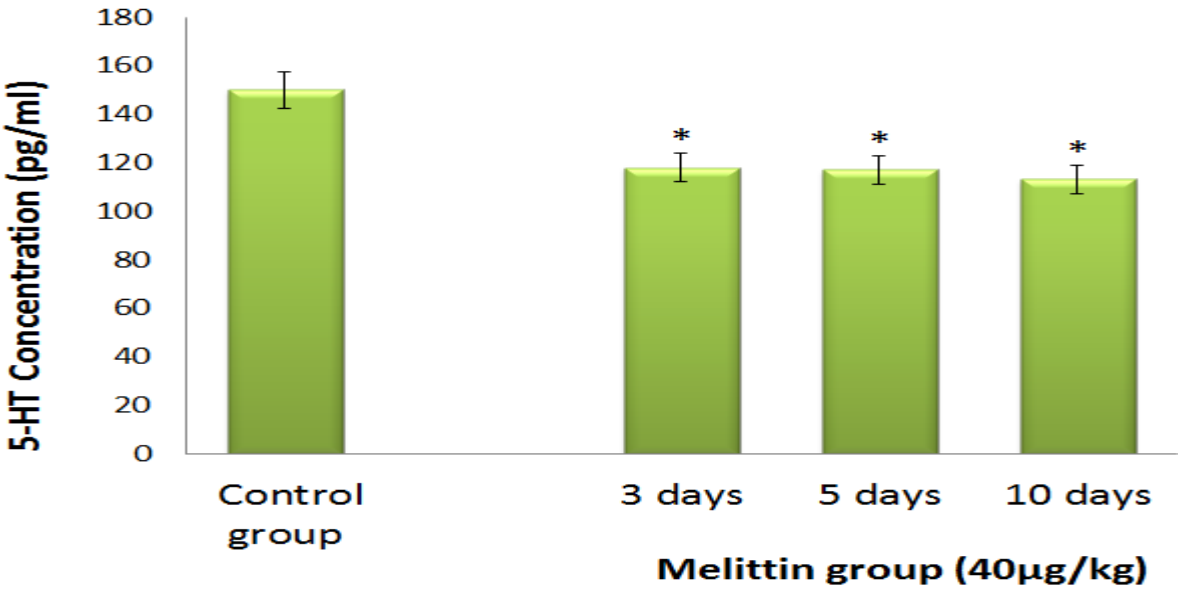

Figure 3. Histogram showing the concentration of 5-HT in melittin group (40 µg/kg) compared to control group. Note that 5-HT concentration in mice treated with melittin (40 µg/kg) for 3, 5 or 10 days was decreased significantly compared to control. $^{*}P< 0.05$.

Table 3. Mean values of 5-HT 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.

Groups	Control Group	Melittin Group 40 µg/kg		
		3 days	5 days	10 days
5-HT Con.	150 ± 4	118 ± 4*	117 ± 4*	113 ± 4*

Figure 4

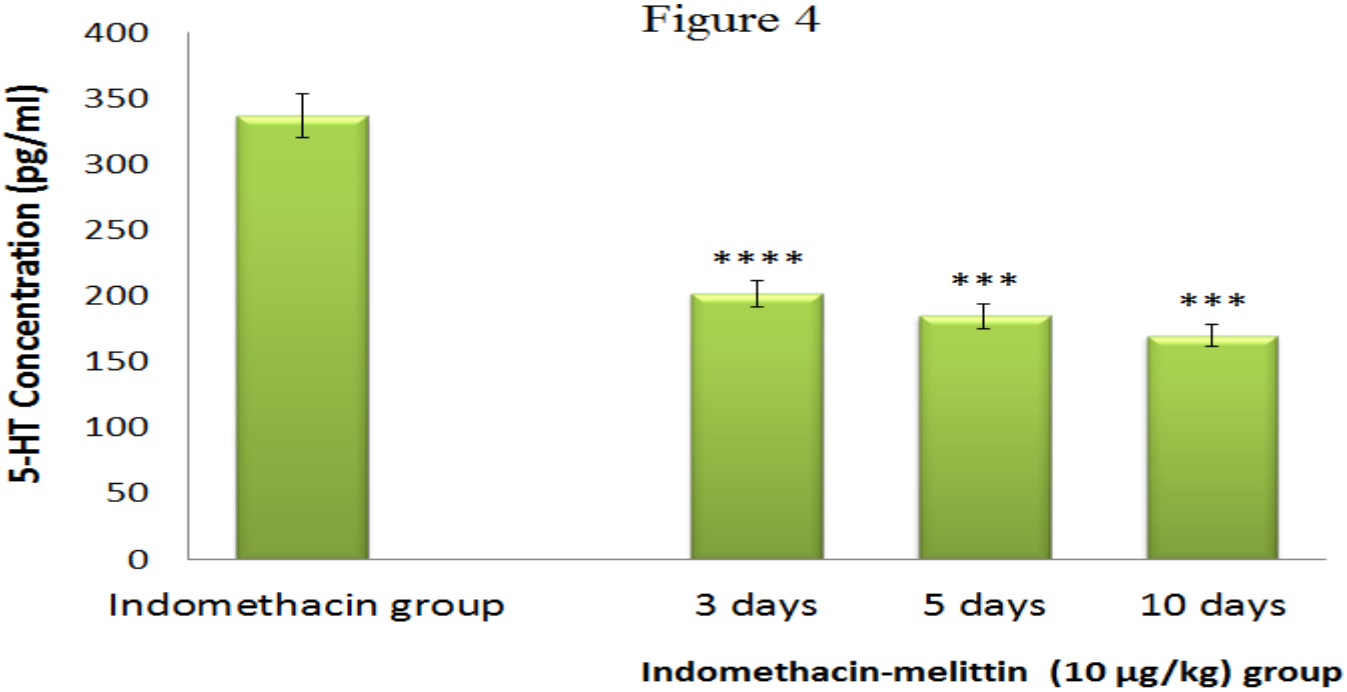

Figure 4. Histogram showing the concentration of 5-HT in indomethacin-melittin (10 µg/kg) group compared to indomethacin group. Note that the mucosal 5-HT concentration in indomethacin-melittin (10 µg/kg) treated jejunum for 3, 5 or 10 days was diminished significantly compared to inflamed group. $^{***}P< 0.001$; $^{****}P< 0.0001$.

Table 4. Mean values of 5-HT concentration ± SE in pg/ml in mucosal tissues of the jejunum in indomethacin-melittin (10 µg/kg) group compared to indomethacin group. $^{***}P< 0.001$; $^{****}P< 0.0001$. Paired-samples *t*-test, *n*=7.

Groups	Indomethacin Group	Indomethacin-Melittin (10 µg/kg) Group		
		3 days	5 days	10 days
5-HT Con.	337 ± 5	202 ± 4****	185 ± 3***	170 ± 3***

treated jejunum for 3, 5 or 10 days was highly significant decreased compared to inflamed group (188±2, 163±2 and 148±1 *vs* 337±5 pg/ml, *P*< 0.0001, 0.001 and 0.002 respectively, *n*=7, Figure 5, Table 5).

However, by using independent samples *t*-test it was found that no significant difference between the mean values of 5-HT concentration in the mucosal jejunum of indomethacin-melittin (10 µg/kg) treated group and indomethacin-melittin (40 µg/kg) treated group when treated for 3 and 5 days, while after 10 days there was a significant difference in the level of 5-HT between both groups (Figure 6, Table 6).

Therefore, it was concluded that treatment with melittin (40 µg/kg) for 10 days was more effective in reducing inflammation through its ability on reducing the level of pro-inflammatory agent (5-HT) compared to those of melittin (10 µg/kg).

DISCUSSION

The present physiological investigations indicated a typical signs of inflammation in jejunum after oral administration of indomethacin. These observations were

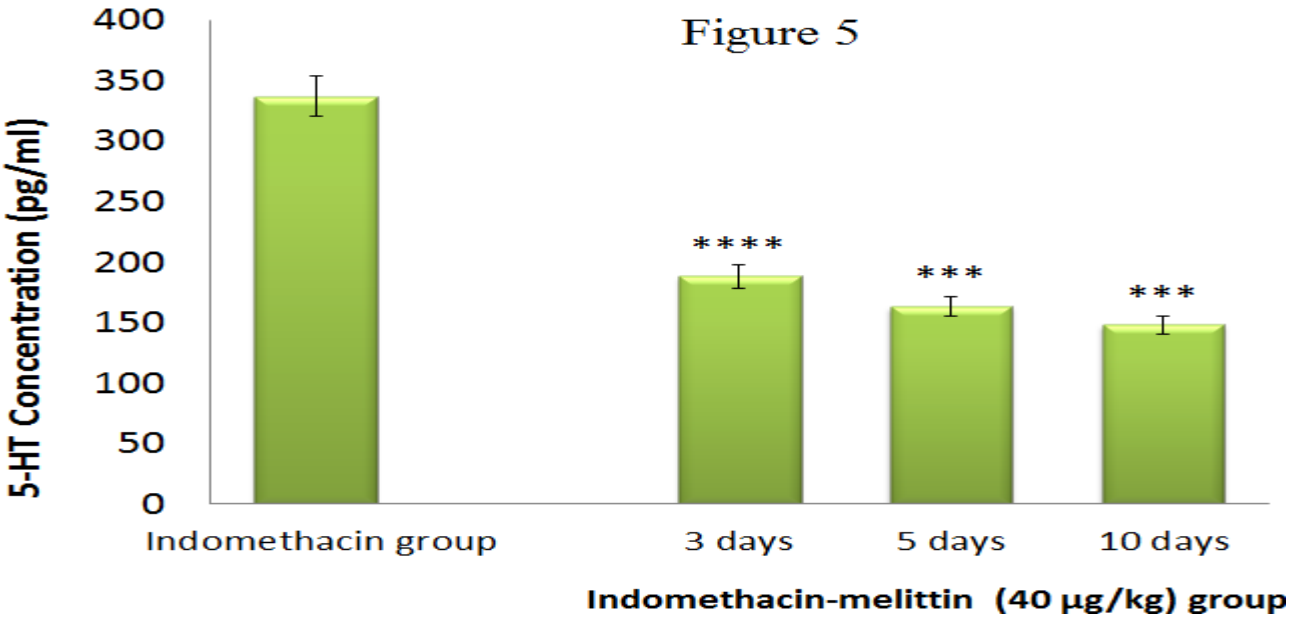

Figure 5. Histogram showing the concentration of 5-HT in indomethacin-melittin (40 µg/kg) group compared to indomethacin group. Note that the level of 5-HT in indomethacin-melittin (40 µg/kg) treated jejunum for 3, 5 or 10 days was highly significant decreased compared to inflamed group. $^{***}P< 0.001$; $^{****}P< 0.0001$.

Table 5. Mean values of 5-HT concentration ± SE in pg/ml in mucosal tissues of the jejunum in indomethacin-melittin (40 µg/kg) group compared to indomethacin group. $^{***}P< 0.001$; $^{****}P< 0.0001$. Paired-samples *t*-test, *n*=7.

Groups	Indomethacin Group	Indomethacin-Melittin (40 µg/kg) Group		
		3 days	5 days	10 days
5-HT Con.	337 ± 5	188 ± 2****	163 ± 2***	148 ± 1***

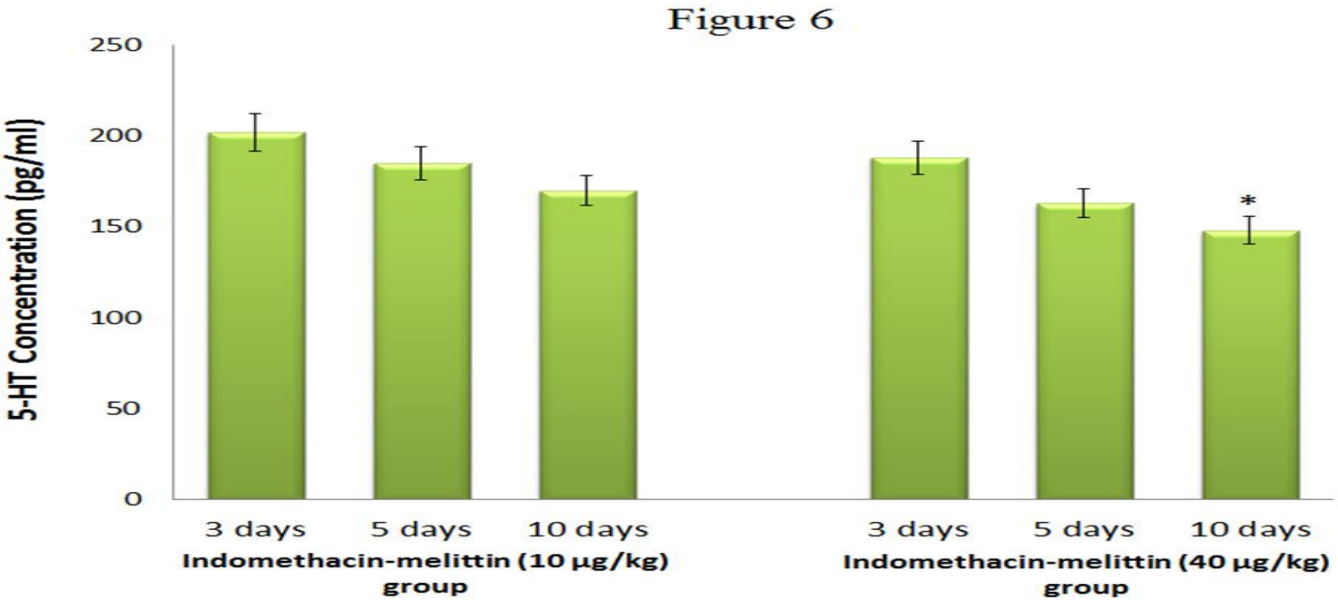

Figure 6. Histogram showing the concentration of 5-HT in indomethacin-melittin (10 µg/kg) group compared to indomethacin-melittin (40 µg/kg) group. Note that no significant differences between the mean values of 5-HT concentration in the mucosal jejunum of indomethacin-melittin (10 µg/kg) group and indomethacin-melittin (40 µg/kg) group after for 3 and 5 days, while after 10 days there was a significant difference in the level of 5-HT between both groups. $^{*}P< 0.05$.

Table 6. Mean values of 5-HT concentration ± SE in pg/ml in mucosal tissues of the jejunum in indomethacin-melittin (10 µg/kg) group compared to indomethacin-melittin (40 µg/kg) group. **P*< 0.05. Independent-samples *t*-test, *n*=7.

Groups	Indomethacin-Melittin (10µg/kg) Group			Indomethacin-Melittin (40µg/kg) Group		
	3 days	5 days	10 days	3 days	5 days	10 days
5-HT Con.	202±4	185±3	170±3	188±2	163±2	148±1*

previously recorded in the stomach (Polat *et al*., 2011), the jejunum (Nandi *et al*., 2010), the ileum (Kubo *et al*., 2010), the small intestine (Silva *et al*., 2012) and in the colon (Pawar *et al*., 2011) in mice treated by indomethacin.

In the present study, oral administration of indomethacin (50 mg/kg/1 day) significantly increased the level of pro-inflammatory agents (5-HT). The inflammatory process in the GI is a key component of mucosal defense against exogenous and endogenous factors (Martin and Wallace, 2006). In the early response to inflammation, 5-HT released from enterochromaffin cells, then act on innate immune cells such as macrophages cells to activate pro-inflammatory cytokine production to increase the resistance to injury and to reduce the severity of GI damage. 5-HT can also act directly on goblet cells to induce mucin production, and on smooth muscle and nerves to alter gut motility (Khan and Ghia, 2010).

The increased level of pro-inflammatory agent (5-HT) was also supported by Khan and Ghia (2010), Imaoka *et al*. (2010) and Nandi *et al*. (2010), who found that indomethacin induced jejunoileitis and caused the early release of 5-HT, where the release of 5-HT contributed at early stages on inflammatory processes in response to the local inflammation in the jejunum (Khan and Ghia, 2010).

On the other hand, Umegaki *et al*. (2010) demonstrated that indomethacin can also frequently induce small intestinal mucosal injury followed by increase production of 5-HT which exerts activation of the anti-inflammatory responses.

The concentration of 5-HT in the mucosal jejunum of mice treated with melittin (10 and 40 µg/kg) for 3, 5 or 10 days was slightly reduced, but it was no significant difference compared to those of control. Our result was in agreement with Stuhlmeier (2007), he mentioned that bee venom and melittin had no inhibitory effects on the pro-inflammatory agents such as 5-HT at a number of cell types. The little reduction of 5-HT concentration in the present study could be attributed to certain conditions such as fear or lack of adaptation of the mice to the laboratory atmosphere (Gray and Green, 1987).

In indomethacin-melittin treated mice, the present study found that the treatment with melittin gradually decreased the concentrations of the pro-inflammatory agent (5-HT) in a dose and time dependent manner, this indicated the effectiveness of melittin in reducing inflammation. These results were confirmed by numerous studies on the effect of melittin on different organs. Moon *et al*. (2007) concluded that the melittin can exert anti-inflammatory effects on many types of cells such as microglia by reduction of pro-inflammatory secretion such as IL-1B, 5-HT, NO and TNF-α. These findings were also supported by the results of Yun *et al*. (2011) who demonstrated that melittin attenuated the inflammation of pancreas by inhibiting the release of pro-inflammatory agents through suppression of NF-κB activity. Park *et al*. (2012) added that melittin provided protection against acute hepatic inflammation through the inhibition of pro-inflammatory agents by preventing the activation of the NF-κB, which induced the release of these agents.

From the above data, it seems that NF-κB plays an important roles in the regulation of inflammatory gene, such as, COX. Therefore, the inhibition of NF-κB activity can be used for treatment of inflammatory diseases (Tak and Firestein, 2001; Kapahi *et al*., 2000), ie the inhibitory effect of melittin on inflammation results from inhibiting the inflammatory stimuli such as IL-1B, 5-HT and TNF-α that induced NF-κB activation via an interaction between melittin and sulfhydryl group of p50 of NF-κB. This data reflected that the inhibition of NF-κB pathway may contribute to the inhibitory effect of melittin on the inflammatory reaction (Park *et al*., 2008). Further evidence showed that the ability of p50 to direct the binding of NF-κB to melittin. These results might indicate that melittin may modify a sulfhydryl group of p50 protein, thereby hindering p50 affinity to the NF-κB binding element (Park *et al*., 2011).

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