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Antinociceptive and Anti-Inflammatory Activity of Bovine Milk-Derived β-Lactoglobulin: Study of Combined Effect with Tramadol or Indomethacin

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Abstract: Considerable efforts and resources have been dedicating to the development of anti-inflammatory products; of particular interest are functional foods capable of modulating the expression of this activity. β-lactoglobulin (β-lag) is the major whey protein of ruminant species and is also present in the milks of many, but not all, other species. This study was designed to evaluate the analgesic and the anti-inflammatory effects of β-lactoglobulin. The antinociceptive effect of Tramadol and β-lag was evaluated either alone or in combination using the hot plate method. Mice received Tramadol alone (20 mg/kg, i.p.), β-lag alone (150, 300 mg/kg, orally), or in combination. The combined treatment was divided into two phases; phase 1(5-60min), showed significant antinociception of combined treatment compared to Tramadol alone. In phase 2 (120-180min.), the antinociceptive effect decreased with time suggesting that combined treatments resulted in synergistic effect in phase 1 and antagonism in phase 2. Carrageenan-induced paw edema was used to investigate the anti-inflammatory activity of β-lag. β-lag alone (150, 300 mg/kg, orally) or combined with Indomethacin (5 mg/kg orally) reduced paw edema, decreased the production of serum PGE2, TNF-α and IL-1β and brain MDA, while it increased brain SOD and TAC. Normal prothrombin time was recorded in β-lag treatment alone while indomethacin decreased prothrombin time. β-lag treatment showed almost normal mucosal layer in histopathological examination of the gastric mucosa compared to indomethacin. β-lag appears to possess anti-inflammatory and peripheral antinociceptive activities, so it may be a candidate for nutraceutical ingredient.

Key words: β-Lactoglobulin · Hot plate · IL-1β · Indomethacin · Inflammation · MDA · Mice · PGE_2 · Prothrombin time · Nociception · Rat, SOD · TAC · TNF-α · Tramadol

INTRODUCTION

Whey protein, a by-product of the cheese-making process, constitutes ~20% of the total bovine milk protein. The components of whey include beta-lactoglobulin, alpha-lactalbumin, bovine serum albumin, lactoferrin, immunoglobulins, lactoperoxidase enzymes, glycomacropeptides, lactose and minerals [1]. Milk proteins are precursors of peptides, which possess various biochemical and physiological properties, including opioid activity, immunomodulatory and cardiovascular effects. These biologically active peptide fragments are released from milk proteins in enzymatic hydrolysis either during gastrointestinal digestion or during fermentation of milk [2]. Alpha-lactorphin (Tyr-Gly-Leu-Phe) and β-lactorphin (Tyr-Leu-Leu-Phe) are tetrapeptides, which correspond the amino acid sequences 50-53 of α-lactalbumin and 102–105 of β-lactoglobulin, respectively. Structurally these tetrapeptides closely resemble endogenous opioid peptides, since the N-terminal amino acid residues in β-endorphin, enkephalins and dynorphin A are Tyr-Gly-

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Gly-Phe-[3]. In fact, both tetrapeptides bind to opioid receptors and show weak opioid activity *in vitro*. α-lactorphin inhibits the contraction of stimulated guinea pig ileum in a naloxone-sensitive manner [3]. β-lactoglobulin is the most abundant protein in milk. β-lactoglobulin reduced dimethylhydrazine-induced colonic aberrant crypt foci formation in rats [4]. β-lag is a rich source of cystein, an essential amino acid that appears to stimulate glutathione synthesis, an anticarcinogenic tripeptide produced by the liver for protection against intestinal tumors in Sprague-Dawley rats [5]. β-lactotensin (His-Ile-Arg-Leu) (f146–149) is an ileum-contracting peptide derived from β-lactoglobulin, which exhibits hypertensive activity. It is a natural ligand for neurotensin NT2 receptors, has an anti-stress effect promotes the abolition of fear memory [6]. β-lag hydrolysate was prepared using thermolysin as proteolytic enzyme released two potent ACE-inhibitory peptides [7, 8]. The biological components of whey demonstrate a range of immune-enhancing properties [9]. In addition, whey has the ability to act as an antioxidant [10], antihypertensive [11], antitumor [12], hypolipidemic [13], antiviral [14], antibacterial [15] and chelating agent [16]. It is well-known that lactoferrin, the minor component of whey proteins, inhibits production of the inflammatory cytokines tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 in monocytes. Lactoferrin protects TNF-α production caused by sensitization of hepatic monocytes (kupffer cells) by lipopolysaccharide [17]. It has been reported that lactoferrin produces analgesia in the thermal, visceral and formalin-evoked nociceptions in rats [18]. However, the efficacy of other proteins has not been clarified with regard to analgesic and anti-inflammatory effect.

Inflammation induced by carrageenan, is acute, non-immune, well-researched and highly reproducible. Cardinal signs of inflammation—edema, hyperalgesia and erythema-develop immediately following subcutaneous injection, resulting from action of pro-inflammatory agents-bradykinin, histamine, tachykinins, complement and reactive oxygen and nitrogen species. Such agents can be generated in situ at the site of insult or by infiltrating cells. Neutrophils readily migrate to sites of inflammation and can generate pro-inflammatory reactive oxygen species [19]. During inflammatory response, several pro-inflammatory mediators are released, including interleukin 1 (IL-1β), IL-6, IL-12, tumor necrosis factor (TNF-α) and interferon (INF-γ) as well as cyclooxygenase-2 (COX-2). These cytokines play major roles in the initiation and amplification of inflammatory processes [20]. Moreover, promotion of procoagulant state, via Thromboxane A2 (TXA2) generated by activated blood platelets and enhanced tissue thrombin production [21]. The inflammatory response is usually quantified by increase in paw size (edema) which is maximal around 5 h post-carrageenan injection and is modulated by inhibitors of specific molecules within the inflammatory cascade. Nonsteroidal anti-inflammatory drugs (NSAIDs) such as indomethacin have a number of adverse side effects, such as gastrointestinal discomfort, inhibition of platelet aggregation and liver and kidney toxicity [22]. The model will continue to be a useful in novel drug development.

The aim of the present study was to assess four points: 1) Oral administration of β -Lag may possess an antinociceptive effect or potentiate the analgesic effect of Tramadol. 2) β-lag can exert a potent anti-inflammatory response alone or potentiate the effect of indomethacin in order to decrease the dose and increase the efficacy of indomethacin that lead to reduce the side effect. 3) How far β –Lag can affect the inflammatory mediators such as prostaglandin E_2, TNF-α and IL-1β. 4) The effect of β -Lag either alone or in combination with indomethacin on brain anti-oxidant activity. 5) The effect of β-lag alone or combination with indomethacin on prothrombin time. 6) The effect of β-lag alone or combination with indomethacin on stomach histopathological status.

MATERIALS AND METHODS

Drugs: Tramadol and indomethacin were obtained from Behringwake Ag, Marburg, Germany. Beta-lactoglobulin was obtained as generous gift from Davisco Foods International (Inc. USA).

Animals: Adult male Sprague Dawley rats weighing 100 – 150 g and Swiss male albino mice weighing 20-25 g were obtained from the animal house of the National Research Center. Animals were housed under standard environmental conditions (23±1°C, 55±5% humidity and a 12-h light: 12h dark cycle) and maintained on a standard laboratory diet and water ad libitum. The guidelines of the ethical care and treatment of the animals followed the regulations of the ethical committee of the National Research Centre, Dokki, Giza, Egypt.

Methods

Assessment of Analgesic Activity: Hot plate test was used to measure the antinociceptive effect of Tramadol in mice based on modifications of a previously described

method [23, 24]. Mice were placed on a hot plate surrounded by a plexiglass cylinder (Model 35100, Ugo Basile, Italy). The temperature of the hot plate was maintained at 55±0.2 °C in an ambient room temperature. Latency to the first sign of discomfort (hind paw licking, withdrawal or jumping) since placement on the heated surface was taken as an index of nociceptive threshold. The reaction time was recorded before and at 5, 15, 30, 60, 120, 180 min after administration of vehicle, or test compounds. Swiss mice were divided into six groups. Group I (control) mice were given distilled water (10 ml/ kg b.wt.). Groups II, III and IV were administered Tramadol (20 mg/kg b.wt, intraperitoneally), β-lag (150, 300 mg/kg body weight), respectively. Groups V and VI were given combined treatment of Tramadol and β–Lag (150, 300 mg/kg body weight) respectively. Hot plate latency values are expressed as means ± SEM.

Induction of Paw Edema: Rats received sub-plantar injections (0.1 ml) of carrageenan (Sigma Chemical Co., St Louis, MO, USA), using a stock concentration of 1% (saline 0.85%), in the left hind paw under brief anesthesia with halothane [25].

Measurement of Paw Edema: Paw thickness was used as a measurement of inflammation induced edema [26]. Paw thickness was measured at several time points (1, 2, 3 and 4 hrs.) using a Dial Caliper (0–150 mm/0.02 mm, Mitutoyo, Japan). The paw thickness was determined at 0 hr (C0: paw thickness before carrageenan injection) and at 1, 2, 3 and 4 hours after carrageenan injection (Ct). Data are expressed as the mean paw thickness ± SEM.

Sprague Dawley rats were divided into seven groups (eight rats each); Group I served as vehicle control group received the respective vehicles only, Group II (carrageenan group): rats injected subcutaneously onto the plantar surface of the right hind paw with 0.1 ml of 1% carrageenan. Group III (indomethacin group): rats received indomethacin in a dose of 5mg/kg body weight orally [27]. Group IV (β-lag 150): rats received β-lag in a dose of 150 mg/kg body weight orally. Group V (β-lag 300): rats received oral β-lag (300 mg/kg). Group VI (indomethacin and β-lag 150): rats received indomethacin (5mg/kg body) and β-lag (150 mg/kg). Group VII (indomethacin and β-lag 300): rats received indomethacin and β-lag (300 mg/kg) orally. All treatment groups were injected with 0.1 ml of 1% carrageenan subcutaneously onto the plantar surface of the right hind paw and one hour before assessment of the anti-inflammatory activity.

Blood Sampling: Four hours after carrageenan injection, the rats were lightly anesthetized with diethyl ether and the blood was collected from the orbital sinus. The blood was divided into two parts. The first part used for determination of prothrombin time. The second part of blood was centrifuged at 2000 rpm for 20 min; the serum was collected and stored at -20°C until use for biochemical analyses.

Determination of Prothrombin Time: Prothrombin time was assayed according to the manufacturer using prothrombin time kit from Stago France. Blood (9vol) is collected in 0.109 M (3.2%) trisodium citrate anticoagulant (1vol), centrifuged 15minutes at 2000-2500g than plasma storage for 8 hours 20 ±5°C.

Biochemical Analyses: Serum tumor necrosis factor-α (TNF- α) was determined according to Seriolo *et al.* [28] by Enzyme-linked Immuno-Sorbent Assay (ELISA) kit (Orgenium Laboratories, Finland). Interleukin-1beta (IL-1β) was determined with an ELISA kit (Endogen Inc, USA) according to Safieh-Garabedian *et al.* [29]. Prostaglandin E2 (PGE2) was determined according to Fitzpatrick *et al.* [30]. The PGE2- ELISA kit was purchased from Orgentec Diagnostika GmbH, Germany.

Preparation of Tissue Homogenate: Brain tissues were removed rapidly, washed in ice-cooled saline, plotted dry and weighed. Brain tissue was homogenized, using a homogenizer (Medical instruments, MPW-120, Poland), with ice-cooled saline to prepare 20% w/v homogenate. The homogenate was then centrifuged at 4000 rpm for 5min at 4°C using a cooling centrifuge to remove cell debris (Laborzentrifugen, 2k15, Sigma, Germany). The aliquot was stored in -80°C till tissue analyses were performed. The aliquot was used for the assessment of brain antioxidant parameters.

Determination of Antioxidant Parameters: The lipid peroxidation marker, malondialdehyde (MDA) was assayed according to the colorimetric method by Mihara and Uchiyama [31]. Estimation of superoxide dismutase (SOD) was done using the method by Nishikimi *et al.* [32]. Finally, total antioxidant capacity (TAC) was assessed colorimetrically using a test reagent kit according to the method described by Koracevic *et al.* [33].

Histopathological Examination: Samples of the stomach were excised and transferred to formalin. The samples were subsequently processed by routine techniques

before embedding in paraffin. Sections were cut at the thickness of 5µm and stained with hematoxylin and eosin (HE). All histopathological changes were observed under a light microscope.

Statistical Analysis: The results are expressed as the mean ± SEM. Data are analyzed with one-way ANOVA. When the variation among groups was proved significant, Tukey's multiple comparison tests are performed to compare significance between groups. The data are analyzed with GraphPad Prism v. 5.0 (GraphPad Software, Inc., CA and USA).Difference is considered significant when p value is < 0.05.

RESULTS

Effect of Analgesic Activity of β-Lag in Mice: Tramadol exerted a significant effect at 60, 120 and 180 min after administration on hot plate latency in mice compared to normal (Table 1). While the analgesic effect of β-lag alone (150 and 300 mg/kg), on hot plate–induced pain in mice, appeared significantly at earlier time points (15 and 30 min) post-administration compared to Tramadol. β-lag antinociceptive effect showed significant increment in hot plate latency with time (60, 120 & 180 min) compared to normal which was not significant from that of Tramadol. Combined treatment of Tramadol with β-lag (150 or 300 mg /kg) potentiated the analgesic effect of either compound alone at early time after administration (5, 15 & 30 min), which showed significant difference from normal and either drugs alone. After 60, 120 and 180 min, the analgesic effect of combined treatment showed remarkable decrease in hot plate latency (Table 1).

Effect of β-Lag on Carrageenan-Induced Paw Edema in Rats: The subplantar injection of carrageenan exerted a significant increase in the paw size (edema) during 4 hours compared to normal rats (Table 2). Indomethacin (5mg/kg) produced a significant anti-inflammatory effect compared to carrageenan control group at 2, 3 and 4 hours post-treatment. β-lag at 150 mg/kg, showed potent anti-inflammatory effect which appeared significantly different from carrageenan and β-lag (300 mg/kg) groups and from indomethacin group at 3 and 4 hours post-treatment. β-lag (300 mg/kg) exerted significant reduction in rat paws edema comparable to carrageenan group at 2 and 4 hours post-treatment. Combination of indomethacin and β-lag (150 mg/kg) improved the anti-inflammatory activity of either drug alone, the reduction in paw edema was significantly different from carrageenan group, indomethacin or β-lag (150 mg/kg) alone, but remained non-significant from normal group at 1, 3 and 4 hours post-treatment. On the other hand, combined administration of indomethacin with β-lag (300 mg/kg) improved the anti-inflammatory effect compared to β-lag (300 mg/kg) alone; the reduction in rat paw edema appeared significantly different from β-lag (300 mg/kg) group at 2, 3 and 4 hours post-treatment.

Effect of β-Lag on Serum PGE2, TNF-α and IL-1β: Carrageenan-induced acute inflammation produced a dramatic and significant increase in serum PGE_2 level and pro-inflammatory cytokines; TNF-α and IL-1β compared to normal control (Table 3). Administration of indomethacin exerted a potent anti-inflammatory effect via significant reduction in serum PGE_2 by 12%, TNF-α by 23% and IL-1β by 57% compared to carrageenan group. β-lag (150 or 300 mg/kg) alone significantly inhibited PGE_2 production by 20%, TNF-α by 30 % and IL-1β by 77% compared to carrageenan-treated rats. Combination of β-lag at either dose with indomethacin exerted a significant reduction in PGE_2 level compared to carrageenan group. The combined treatment of β-lag (150mg/kg) with indomethacin caused significant decrease in PGE_2 level compared to indomethacin alone. Regarding serum levels of TNF-α and IL-1β, the combined treatment with β-lag (150, 300 mg/kg) and indomethacin showed significant inhibition in elevation of pro-inflammatory cytokines comparable to carrageenan and indomethacin groups that recorded 30% and 82 % reduction in serum TNF-α and IL-1β, respectively compared to indomethacin alone.

Effect of β-Lag on Brain Antioxidant Status in Rats: Inflammation induced by carrageenan for four hours resulted in significant increase of brain lipid peroxidation marker, MDA, while significantly decreased brain total antioxidant capacity (TAC) and superoxide dismutase (SOD) activity compared to normal control (Table 4). Treatment with indomethacin (5m/kg) showed no significant difference in brain MDA level, while significantly inhibited the decrement in TAC and SOD activity compared to carrageenan group. β-lag (150 or 300 mg/kg) showed significant decrease in the brain MDA level by 74%, 65% respectively, compared to carrageenan group. Moreover, β-lag at both doses increased significantly brain TAC and SOD activity compared to indomethacin alone and carrageenan-treated rats. In addition, combination of β-lag (150 or 300mg/kg) with indomethacin significantly decreased brain MDA level by

Table 1: Effect of β-lag (150 or 300mg/kg, p.o) alone or in combination with Tramadol (20 mg/kg, i.p) on hot plate latency in mice

	Hot plate latency (sec)					
Time after drug administration (min)	Control	Tramadol	β-lag 150	β-Lag300	Tramadol + β-lag 150	Tramadol + β-lag 300
5	11.33 ± 0.49^{A1}	12.83 ± 0.75^{A1}	12.50 ± 0.22^{A1}	13.83 ± 1.01^{A1}	22.33 ± 1.05^{B1}	23.17 ± 0.75^{B1}
15	11.67 ± 0.56^{A2}	14.67 ± 0.67^{A2C2}	19.50 ± 0.43^{B2}	15.83 ± 1.08^{C2}	26.67 ± 0.67^{D2}	23.00 ± 1.26^{B2}
30	11.17 ± 0.48^{A3}	13.67 ± 0.67^{A3}	21.50 ± 1.06^{B3}	19.33 ± 0.49^{B3}	28.00 ± 1.24^{C3}	21.00 ± 1.00^{B3}
60	11.83 ± 0.65^{A4}	19.33 ± 0.84^{B4D4}	$22.0\pm.03^{B4C4}$	19.67 ± 0.88^{B4D4}	24.67 ± 1.76^{C4}	15.67 ± 0.80^{A4D4}
120	11.67 ± 0.49^{A5}	23.17 ± 1.08^{B5}	23.33 ± 0.71^{B5}	18.33 ± 0.76^{C5D5}	19.67 ± 0.21^{C5}	15.83 ± 0.70^{D5}
180	12.00 ± 0.58^{A6}	24.50 ± 1.12^{B6}	24.83 ± 1.28^{B6}	21.17 ± 0.60^{B6C6}	19.67 ± 1.71^{C6D6}	15.67 ± 0.49^{A6D6}

Data are expressed as mean ± SEM (n=8). Statistical analyses were carried out by one way ANOVA followed by Tukey's Multiple Comparison Test. Groups with different superscripts and their numbers are significantly different at $p< 0.05$. Groups with similar superscripts and their numbers are non-significantly different at $p<0.05$. Each number (1, 2, 3 and 4) refers to the time after Drug administration.

Table 2: Effect of β-lactoglobulin (150 or 300mg/kg, p.o) alone or in combination with indomethacin (5mg/kg, p.o) on carrageenan- induced paw edema in rats

	Paw Edema Thickness (mm)			
Groups	1 hours	2 hours	3 hours	4 hours
Control	3.63 ± 0.08^{A1}	3.65 ± 0.04^{A2}	3.68 ± 0.07^{A3C3}	3.63 ± 0.06^{A4}
Carrageenan	5.15 ± 0.23^{B1}	5.75 ± 0.21^{B2}	6.23 ± 0.27^{B3}	6.90 ± 0.19^{B4}
Indomethacin	4.77 ± 0.21^{B1C1}	2.85 ± 0.20^{D2}	3.97 ± 0.14^{A3}	4.67 ± 0.15^{D4}
β- lag 150	3.95 ± 0.08^{A1C1}	4.07 ± 0.13^{A2}	4.27 ± 0.15^{A3}	4.28 ± 0.19^{A4D4}
β- lag 300	4.58 ± 0.20^{B1C1D1}	4.85 ± 0.16^{C2}	6.65 ± 0.19^{B3}	5.45 ± 0.15^{C4}
Indo+ β- lag 150	3.87 ± 0.13^{A1D1}	2.47 ± 0.14^{D2}	3.28 ± 0.11^{C3}	3.80 ± 0.10^{A4}
Indo+ β-lag300	3.90 ± 0.36^{A1C1}	2.58 ± 0.12^{D2}	3.97 ± 0.21^{A3C3}	4.57 ± 0.20^{D4}

Data are expressed as mean ± SEM (n=8). Statistical analyses were carried out by one way ANOVA followed by Tukey's Multiple Comparison Test. Groups with different superscripts and their numbers are significantly different at $p< 0.05$. Groups with similar superscripts and their numbers are non-significantly different at $p< 0.05$. In addition, each number (1, 2, 3 and 4) refers to the time of paw edema determined.

Table 3: Effect of β-lag (150 or 300 mg/kg, p.o) alone or in combination with indomethacin (5mg/kg, p.o) on serum PGE_2, TNF-α and IL-1β in carrageenan – induced inflammation in rats

Group	Serum PGE2 (ng/l)	Serum TNF-α (ng/l)	Serum IL-1β (ng/l)
Control	67.68 ± 0.94^{AD}	60.50 ± 0.86^{AC}	2.70 ± 0.16^{A}
Carrageenan	82.25 ± 1.42^{B}	85.00 ± 1.37^{B}	27.85 ± 0.15^{B}
Indomethacin	72.75 ± 1.52^{C}	65.33 ± 1.15^{C}	12.04 ± 0.56^{D}
ß-Lag 150	65.83 ± 0.83^{A}	59.25 ± 0.96^{A}	6.35 ± 0.12^{C}
ß-Lag 300	65.16 ± 0.82^{A}	60.08 ± 0.52^{A}	6.13 ± 0.09^{C}
Indo+ ß-Lag(150)	66.33 ± 0.71^{A}	45.50 ± 1.62^{D}	2.14 ± 0.06^{A}
Indo+ ß-Lag(300)	71.87 ± 0.96^{DC}	58.50 ± 0.98^{A}	2.44 ± 0.11^{A}

Data are expressed as mean ± SEM (n=8). Statistical analyses were carried out by one way ANOVA followed by Tukey's Multiple Comparison Test. Groups with different superscripts are significantly different at $p< 0.05$. Groups with similar superscripts are non-significantly different at $p< 0.05$.

Table 4: Effect of β-lag (150 or 300 mg/kg, p.o) alone or in combination with indomethacin (5 mg/kg, p.o) on brain MDA, TAC, SOD and plasma PT after carrageenan–induced inflammation in rats

Group	Brain MDA (nmol/g tissue)	Brain TAC (mmol/mg tissue)	Brain SOD (nmol/g tissue)	Plasma (PT) (Sec)
Control	6.62 ± 0.14^{A}	352.83 ± 7.07^{A}	328.33 ± 2.55^{A}	23.83 ± 0.60^{A}
Carrageenan	25.43 ± 0.60^{B}	63.33 ± 4.41^{B}	91.83 ± 1.89^{B}	17.83 ± 0.60^{B}
Indomethacin	24.05 ± 1.10^{B}	170.00 ± 3.42^{E}	189.33 ± 2.06^{E}	22.83 ± 1.25^{A}
ß-Lag 150	6.62 ± 0.18^{A}	271.67 ± 6.01^{C}	315.50 ± 1.77^{C}	24.00 ± 0.52^{A}
ß-Lag 300	9.00 ± 0.25^{A}	325.00 ± 5.63^{D}	461.67 ± 2.74^{D}	23.00 ± 0.97^{A}
Indo+ ß-Lag(150)	17.92 ± 0.57^{D}	205.00 ± 4.28^{F}	226.83 ± 2.20^{F}	6.83 ± 0.48^{C}
Indo+ ß-Lag(300)	20.17 ± 0.31^{D}	251.67 ± 4.77^{C}	278.83 ± 3.11^{G}	10.83 ± 0.48^{D}

Data are expressed as mean ± SEM (n=8). Statistical analyses were carried out by one way ANOVA followed by Tukey's Multiple Comparison Test. Groups with different superscripts are significantly different at $p< 0.05$. Groups with similar superscripts are non-significantly different at $p< 0.05$

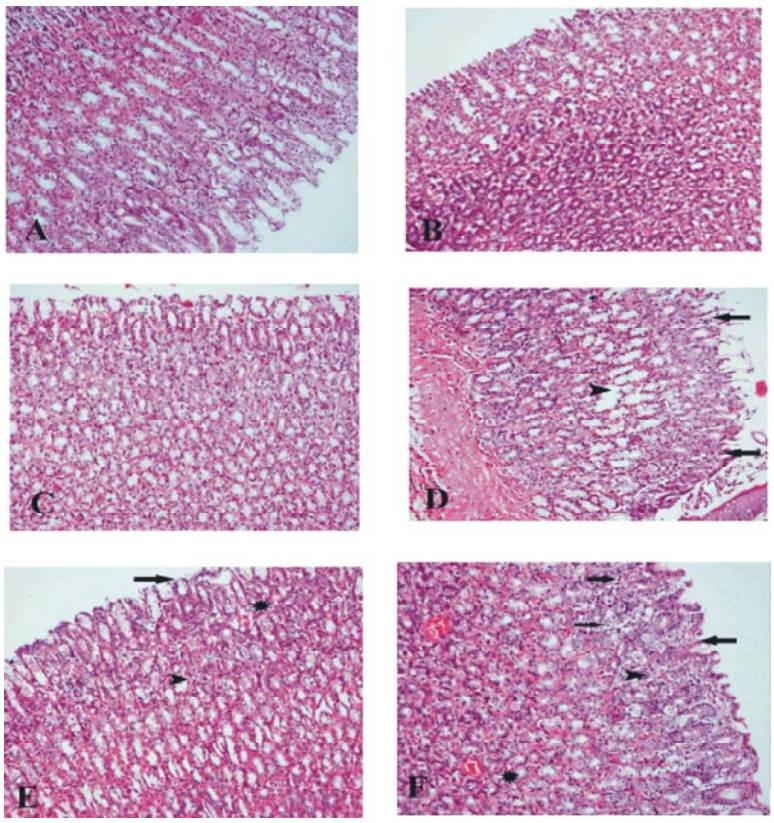

Fig. 1: Hematoxylin-eosin stained stomach sections (X 200). A: Photomicrograph of section from the stomach of normal rat showing normal histological appearance mucosa. B: Section from the stomach of rat treated with β- lag (150 mg/kg) showing normal histological mucosal appearance. C: Section from the stomach of rat treated with β-lag (300 mg/kg) showing normal histological mucosal appearance. D: Section from the stomach of rat treated with indomethacin showing erosion of mucosa (long arrow), degeneration and necrosis of gastric gland and haemorrhage (arrowhead). E: Section from the stomach of rat treated with indomethacin and β-lag (150 mg/kg) showing nearly normal gastric mucosa with slight leukocytic infiltrations (arrowhead) and haemorrhage (star). F: Section from the stomach of rat treated with indomethacin and β-lag (300 mg/kg) showing erosion of mucosa (long arrow) with degeneration and necrosis of gastric gland (arrowhead) and haemorrhage (star).

30% and 20%, respectively as compared to indomethacin alone and carrageenan group. Further, brain antioxidant status was improved in combined treatment of β-lag (150 or 300 mg/kg) with indomethacin as observed by significant elevation in brain TAC and SOD compared to indomethacin alone after four hours of carrageenan injection.

Effect of β-Lag on Plasma Prothrombin (PT) in Carrageenan-Induced Inflammation: The inflammatory response evoked by carrageenan significantly decreased prothrombin time (PT) compared to normal group (Table 4). Treatment with indomethacin (5m/kg) showed significant increase the plasma PT by 72% as compared to carrageenan group. Similarly, treatment with β-lag (150 or 300 mg/kg) alone showed significant increase the plasma PT by 65% and 70% respectively. Effect of single treatments was non-significantly different from normal control. While, combination of β-lag (150 or 300 mg/kg) with indomethacin exerted significant decrease in plasma PT by 38% and 60%, respectively as compared to carrageenan group.

Histopathological Examination: The histological appearance of the stomach in the control group (Fig. 1A) was normal. Sections of gastric mucosa from beta-lactoglobulin (β-lag 150 or 300 mg/kg) group showed an almost normal mucosal layer (Fig. 1B &1C). Indomethacin (5 mg /kg)-treated group showed mild to moderate gastric mucosal injury and mucosal erosion associated with

diffused leucocyte cell infiltration was observed in lamina propria of the mucosal layer (Fig. 1D). Rats treated with indomethacin and β-lag (150 mg/kg) maintained the normal histological structure of glandular portion with reduced leucocyte cell infiltration in the gastric mucosa (Fig. 1E). Indomethacin and β-lag (300 mg/kg) showed mild reduction in haemorrhagic lesions of gastric mucosa induced by indomethacin (Fig. 1F).

DISCUSSION

Combining analgesics into a single product may facilitate prescribing and compliance by reducing the total number of medications that a patient must take to manage pain. Combining products with different mechanisms of action may also provide multimodal coverage of a broad spectrum of pain and, in addition, enable the individual agents potentially to act in a greater than additive (synergistic) fashion. Furthermore, in terms of safety, lower doses of each individual analgesic, used in the combination, may result in a lower incidence of individual adverse events [34]. Tramadol was initially described as a traditional opioid; however, Tramadol has low affinity for μ-opioid receptors, more akin to the non-opioid imipramine than the opioid codeine [35]. The M1 metabolite of Tramadol binds μ-opioid receptors more strongly than the parent drug and an opioid component contributes to Tramadol-induced antinociception (animals) and analgesia (humans). However, because the opioid antagonist naloxone does not reverse all of the antinociceptive [35] or analgesic [36], effects of Tramadol, additional mechanisms must contribute significantly to its analgesic effects. Several studies have shown that Tramadol inhibits re-uptake of serotonin and norepinephrine, which synergistically enhances the opioid mechanism of action [37, 38]. It may also explain why Tramadol is effective in opioid-resistant chronic pain states [39] and other painful conditions [40, 41]. Because current therapy for pain relief is inadequate for some patients and chronic pain is difficult to treat, the search for new analgesic compounds or therapies appeared to be a must.

Whey proteins contain opioid-like sequences in their primary structure, namely α-lactalbumin (α-la) (both bovine and human) f (50–53) and β-lactoglobulin (β-lag) (bovine) f (102–105). These peptides have been termed α- and β-lactorphins. Proteolysis of α-lactalbumin with pepsin produced α-lactorphin; whilst digestion of β-lactoglobulin with pepsin and then with trypsin, or with trypsin and chymotrypsin, yielded β-lactorphin. α-lactorphin exerts weak but consistent opioid activity in the guinea pig ileum and in connection with receptor-binding; whereas β-lactorphin-despite its similar receptor-binding affinity-exerts an apparent non-opioid stimulatory effect on guinea pig ileum [42]. These peptides show very low affinity for opioid receptors and are μ-type receptor ligands. Both α- and β-lactorphin were found to displace ^{3}H-naloxone from its binding sites at micromolar concentrations. The reference opioid peptide, morphine, displaced ^{3}H-naloxone in the nanomolar range (IC_{50}=23±13 nM). Furthermore, it was shown that digestion of β-lag with chymotrypsin produced His-Ile-Arg-Leu (β-lactotensin, β-lag f (146–149)). The pharmacological activity of β-lactotensin was similar to that of β-lactorphin, as they induced stimulation that was antagonised neither by naloxone nor by atropine [42, 43]. As combining analgesics with different mechanisms or sites of action can allow reducing doses of the component drugs, reducing overall adverse effects with comparable analgesia. Likewise, combining short-acting and long-acting agents can result in both shorter onset and longer duration of analgesia. The analgesic activity of Tramadol when combined with β-lactoglobulin (150 and 300 mg/kg), in the current protocol, produced early antinociceptive effect in mice using hot-plate method after 5 min of treatment. Antinociceptive response to Tramadol alone showed delayed effect starting from 60 min after treatment. Tramadol analgesic effect occurred within approximately 1 h of dosing, consistent with the pharmacokinetic profile of the parent drug, whereas the maximum effects of Tramadol appeared later (within approximately 2.5 h) [44]. β-lag (150 and 300 mg/kg) showed early onset of antinociceptive activity after 15 min while that of Tramadol appeared after 60 min. The analgesic effect of either compounds tended to increase in a time-dependent manner, whereas combined effect which appeared at early time point (5 min), decreased remarkably at 60 min-interval, with the same latencies after 120 and 180 min during which high latencies were reported to individual treatments. The exact mechanism of such alteration in combined treatment warrants further investigation.

Carrageenan-induced paw edema in mice has been accepted as a useful phlogistic tool for investigating anti-inflammatory agents. There are biphasic effects in carrageenan induced edema. Carrageenan induced paw edema is believed to be biphasic, of which the first phase is mediated by the release of histamine and 5-hydroxytryptamine in the early stage followed by kinin release and then PG in the later phase [45]. Edema is one

of the fundamental actions of acute inflammation and is an essential parameter to be considered when evaluating compounds with potential anti-inflammatory activity [46]. β- lag showed effectively inhibitory activity on carrageenan-induced paw inflammation over a period of 4 hours. The anti-inflammatory activity of β-lag is clearly observed in carrageenan-induced rat paw edema, in both 150 and 300 mg/kg in oral treatment models. When combined with indomethacin, β-lag produced greater reduction in carrageenan-induced edema than β-lag or indomethacin alone, throughout 4 hours of treatment. Whey proteins reported earlier an anti-inflammatory effect in carrageenan-induced paw inflammation [47], alpha-lactalbumin Orally administered alpha-lactalbumin showed inhibition of writhing induced by acetic acid in mice; suppression of nociception and inflammation in rat footpads caused by carrageenan in rat; and therapeutic effects on the development of adjuvant-induced pain and inflammation in rat [48] and minor component lactoferrin produces analgesia in the thermal, visceral and formalin-evoked nociceptions in rats [18]. Inflammation is the first response of the immune system to infection or irritation; It is mediated by cytokines such as TNF-α, IL-1β, IL-2, IL-6 and PGE2 [49]. Inhibitors of these cytokines, such NSAIDs (e.g.) Indomethacin, currently are the choice of anti-inflammatory agents. However, drugs are not free of side effects, which pose potential harm in the risk of other unwanted diseases. The search for natural products with anti-inflammatory activity has increased markedly in recent years [50]. The animal model of carrageenan induced-acute inflammation was associated with marked accumulation of COX mRNA and thromboxane (TXA_2) with coincident local production of PGE_2, moreover, PG production was associated with upregulation of COX-2 mRNA and protein in the affected paws the affected paws [51]. Non-steroidal anti-inflammatory drugs (NSAIDs) alleviate pain by counteracting the COX enzyme [52]. On its own, COX enzyme synthesizes prostaglandins, creating inflammation. NSAIDs prevent the prostaglandins from ever being synthesized, reducing or eliminating the pain. COX-2 selective inhibitor is a form of NSAID that directly targets COX-2, an enzyme responsible for inflammation and pain. Selectivity for COX-2 reduces the risk of peptic ulceration. It has been reported that COX-2-selectivity does not affect other adverse effects of NSAIDs (most notably an increased risk of renal failure) [53]. During current study, oral doses of β-lag (150 and 300 mg/kg) notably inhibited the elevation in serum PGE_2 and when combined with indomethacin more significant reduction in serum PGE_2 was recorded specially with β-lag (150 mg/kg) than indomethacin alone. The data suggest COX-2 selective inhibition as a potential mechanism of the anti-inflammatory effect of β-lag alone or in combination with NSAID; indomethacin and hence most probable less gastrointestinal side-effects. As a consequence of shutting down the cyclooxygenase pathway, the accumulation of AA and the products from lipoxygenase can induce up-regulation of pro-inflammatory cytokines at transcriptional and post-transcriptional levels through the nuclear factor NF-kB pathway [54]. Much evidence supports the notion that the cytokines, TNF-α and IL-1β are mediators of chronic pain states produced by inflammation or sensory nerve damage. In animal models of inflammatory or neuropathic pain, the levels of these cytokines are elevated in the spinal cord and at the site of injury [55,56], Peripheral or central administration of TNF-α or IL-1β results in hyperalgesia [57], whereas inhibiting the actions of cytokines attenuates hyperalgesia induced by peripheral inflammation, neuropathy, or cytokine administration [58,59], This sensitization is attenuated by inhibitors selective for the inducible form of cyclooxygenase, COX-2, suggesting that the excitatory effects of cytokines in sensory neurons are dependent on the production of prostaglandins and/or thromboxanes. This is consistent with previous observations where COX-2 inhibition attenuated cytokine-induced hyperalgesia [60]. Recently, TNFα and IL-1β increase COX-2 expression and PGE_2 production in Dorsal Root Ganglia (DRG) and suggest that cytokine-induced sensitization of sensory neurons is secondary to prostaglandin production [61]. Consistent with previous observations, treatment of animals with acute inflammation with β-lag (150 and 300 mg/kg) produced remarkable inhibition in serum TNF-α and IL-1βafter 4 hours of treatment. When administered with indomethacin, potent reduction in TNFα and IL-1βlevels was recorded compared to either compound alone. The notion strongly supports that β-lag may possess potent anti-inflammatory effect via COX-2 pathway inhibition as indicated previously that COX-2 but not COX-1 is upregulated in paw tissue concomitant to the development of edema in Lewis rat model of adjuvant-induced arthritis and that selective inhibition of COX-2 rapidly reverses PG production and edema in arthritic paws. In addition, selective e inhibition of COX-2 activity downregulates COX-2 and IL-6 production in arthritic paws and suppresses the systemic production of IL-6 induced by adjuvant [51]. Therefore, such results revealed that β–lag at two doses alone and combination displayed

significant anti-inflammatory activities in carrageenan-induced paw edema, which might be related to the reduction of PGE2, TNF-α and IL-1β.

Moreover, during the inflammatory process there is an increased burst of ROS in several different inflammatory cells, caused by the highly specialized NADPH- dependent oxidase system [62]. The ROS such as O2●- radicals, are converted to powerful oxidizing radicals like hydroxyl radical (●OH), alkoxy radicals (RO●), peroxyl radicals (ROO●), some of the radical species are converted to molecular oxidants like hydrogen peroxide (H_2O_2) [63]. The carrageenan-induced inflammatory response has been linked to the neutrophil infiltration, the release of neutrophil-derived mediators, as well as the production of neutrophil-derived free radicals, such as hydrogen peroxide, superoxide and hydroxyl radicals [64]. The production of MDA is due to the attack of plasma membranes by free radicals [65]. Administration of β-lag in both dose either alone or in combination with indomethacin group exerted potent anti-oxidative properties than indomethacin alone as reported by reduction in MDA level, increase in total antioxidant capacity (TAC) and superoxide dismutase (SOD) activity in brain tissue of inflamed rats. This antioxidant effect appeared significantly after 4 hr of administration in dose dependent manner. SOD2 is the most prominent and widely distributed form of the SOD family and plays a critical role in modulating the production of inflammatory mediators via its antioxidant defensive properties [66]. It is expected that the potential effect β-lag of in preventing further accumulation of free radicals as well as oxidative stress could be due to their anti-oxidant capacity and/or their anti-inflammatory effect. High level of SOD_2 inhibits the over-expression of PLA_2 and downstream PGE_2 production via nuclear factor–kappa B (NF-kB)-dependent pathway [67] and thereby abrogates the development of inflammation. The up-regulation of SOD2 following inhibition of COX-2 by the rofecoxib or ibuprofen treatment during acute inflammation contributed to the anti-inflammatory and analgesic effects via affecting the activation of phospholipase A2 in the arachidonic acid pathway [68].

An important contribution to haemostatic changes observed following inflammation is given by the "acute phase reaction that represents a highly complex reaction of the organism to a variety of injuries, aimed to restore homeostasis. One important feature of the "acute phase reaction" is the hepatic synthesis of proteins involved in the coagulation cascade [69]. However, changes in the haemostatic balance caused by the "acute phase reaction" may lead to an increased risk of thrombosis, both in human and in experimental animals [70]. Carrageenan-induced rat paw oedema remains localized in the injection area. Little is known about the systemic reaction to the local injection of carrageenan in the rat. In the past, studies carried out in order to evaluate the systemic response to carrageenan oedema induction in rats have only analyzed platelet function, without taking in account any coagulation parameter [71]; thus, there is no enough information on the impact of local acute inflammation on the haemostatic balance. Investigating the effect of acute phase inflammation on prothrombin time, which could only reflect the increase in coagulation factors involved in the extrinsic pathway (factors VII and tissue factor). Although it is known that inflammation causes tissue factor generation and hence increased activation of factor VII [72, 73], we did not measure plasma tissue factor levels in our model, carrageenan-induced local acute inflammation that is resolved in about 4 h triggers a systemic reaction, mainly characterized by a shortened prothrombin time value, that might lead toward a pro-thrombotic state and further highlight the important link between inflammation and haemostasis. Accordingly, in the late phase of carrageenan-induced rat paw oedema; i.e. 24 h following carrageenan injection, when there is an almost complete absence of local inflammatory symptoms. There were an increased plasma fibrinogen levels, antithrombin III activity and serum interleukin-6 levels, concomitant to a shortened prothrombin time and to increased platelet responsiveness to ADP [74]. Hence, our results demonstrate that a haemostatic imbalance occurs following carrageenan-induced rat paw oedema. Upon treatment with either indomethacin or β- lag, prothrombin time reached its normal values after 4h of treatment while combined treatment dramatically and unexpectedly shortened prothrombin time to values which exceeded that in carrageenan treated animals. This observation warrants further investigation.

Nonsteroidal anti-inflammatory drugs (NSAIDs), including indomethacin, are effective anti-inflammatory and analgesic agents commonly used in the treatment of rheumatoid arthritis and osteoarthritis. NSAIDs inhibit PG formation through inhibition of both the COX-1 and COX-2 enzymes [75]. Longterm NSAID treatment is often limited, however, by gastrointestinal ulcerogenicity that may result from the suppression of physiological PG production in these tissues. The ability of a selective COX-2 inhibitor to block PG production (and acute tissue inflammation [76]. *In vivo* at dosages that do not affect stomach PG production, suggesting that COX-2 inhibitors

may provide a safer therapeutic alternative to NSAIDs. In the present study, indomethacin (5mg/kg) caused mucosal injury via local irritating and systemic effect. The later is mediated through cyclooxygenase inhibition. COX-1 which is constitutively expressed and plays important role in maintenance of normal gastric mucosa and other organs functions and COX-2, which is the inducible form, is up regulated in areas of inflammation [77]. So, indomethacin produced gastric mucosal lesions due the reduction in the cytoprotective, PGE2, following neutrophil infiltration as exogenous administration of PGE2 produced an anti-ulcer effect by preventing the indomethacin-induced TNF-α increase [78]. In addition, the inhibition of indomethacin-induced gastric lesions is thought to be related to the antioxidant effect [79]. As supported by the combined treatment of indomethacin and two doses alone (150, 300mg/kg), which markedly counteracted the histological alterations induced by indomethacin. This can be attributed to their antioxidant, which significantly reduced the oxidative stress threat leading to reduction in pathological changes and restoration of normal physiological of stomach. Moreover, it has been reported that alpha-lactalbumin (another major whey protein) fortifies the mucus gel layer by stimulating mucin production and secretion in gastric mucus-producing cells and that this enhancing effect is independent of endogenous PGE2. Alpha-lactalbumin stimulates mucin synthesis and secretion in mucusproducing cells and induces increased thickness of the mucus gel layer in the gastric mucosa, suggesting that stimulation of mucus metabolism by alpha- Lactalbumin contributes to its gastroprotective actions [80]. From the current data, β-lag also possess a gastroprotective activity via its anti-inflammatory and anti-oxidative properties.

CONCLUSION

Our study highlights the therapeutic potential of combining medications with different mechanisms of action, particularly an opioid (Tramadol) or a nonsteroidal anti-inflammatory drug (NSAID) with natural compound (beta-lactoglobulin), which may improve clinical outcomes under certain conditions with the use of a combination of analgesics and/or anti-inflammatory drugs, rather than reliance on a single agent. A combination is most effective when the individual agents act through different mechanisms and act synergistically. By activating multiple pain-inhibitory pathways, combination therapy can provide more effective pain relief for a broader spectrum of pain and might also reduce adverse drug reactions.

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