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Gastroprotective Effect of L-Carnitine on Indomethacin-Induced Gastric Ulcer in Rats: The Involvement of Antioxidant Mechanisms and Nitric Oxide

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Abstract

This study investigated the gastroprotective effect of L-carnitine against indomethacin-induced gastric ulcer in rats and the possible mechanisms underlying this effect. Two sets of experiments were performed. In the first set male albino rats (170-210 g) were randomly allocated into a normal control group, ulcer control group (received a single dose of indomethacin 30 mg/kg po) and three ulcer groups pretreated with ranitidine (50 mg/kg po), which was used as a standard anti-ulcer agent, and L-carnitine in two dose levels (100, and 300 mg/kg po) respectively 1 h before ulcer induction. In the second set, male rats were randomly assigned into a normal control and ulcer control groups as in the first set, a third group received N G-nitro-L-arginine-methyl ester (L-NAME) 50 mg/kg ip 1/2 h before indomethacin administration. A fourth and a fifth group received L-carnitine (300 mg/kg po) 1 hour before ulcer induction with indomethacin. The fifth group received in addition L-NAME (50 mg/kg ip) 1/2 h before L-carnitine. The animals were killed 4 h after indomethacin administration and the mucosal tissue was used for gastric injury evaluation both macroscopically and biochemically. Indomethacin produced severe ulceration together with disturbed redox state manifested by elevated malondialdehyde (MDA) level and lowered reduced glutathione (GSH) content. There is also an increase in myeloperoxidase (MPO) activity, an index of neutrophil infiltration, together with decreased gastric mucosal nitric oxide (NO) content measured as nitrite. Both ranitidine and L-carnitine, significantly ameliorated the indomethacin-induced gastric lesions as manifested by a significant reduction in ulcer index, with the effect of L-carnitine being dose dependent. This gastroprotective effect was associated with a significant decrease in MDA, increase in GSH, reduction in MPO activity and restoration of gastric mucosal nitrite level to normal control values. Administration of L-NAME significantly exacerbated gastric lesions and reduced the gastroprotective effect of L-carnitine as well as the gastric mucosal nitrite concentration, without altering the effect of L-carnitine on MDA or GSH. These results suggest that L-carnitine has a dose-dependent gastroprotective effect against indomethacin induced ulcer which is comparable to the H2 antagonist ranitidine. This gastroprotective effect is probably due to several mechanisms that include antioxidant activity, inhibition of leukocyte infiltration in the gastric lesion, increasing the availability of NO in the gastric mucosa through reduction of reactive oxygen species (ROS) and increasing the enzymatic production of NO from cNOS, probably due to a decreased oxidative uncoupling of the enzyme.

Key Words: *L-carnitine – Indomethacin – Gastric ulcer – Antioxidants – Neutrophil infiltration – Nitric oxide.*

Introduction

NON-STEROIDAL anti-inflammatory drugs (NSAIDs) such as indomethacin are capable of producing injury to gastrointestinal mucosa in experimental animals and humans and their use is associated with a significant risk of hemorrhage, erosions, and perforation of gastric ulcers **[1]**. The molecular basis for the gastric toxicity of NSAIDs is widely believed to be mainly due to their non-selective inhibition of cyclooxygenase and consequent reduction in prostaglandins synthesis **[2,3]**. However, it has been reported that reactive oxygen species (ROS) produced by leukocytes recruited and activated after indomethacin treatment have a crucial role in gastrointestinal mucosal injury via ROS mediated oxidation of important biomolecules such as lipid, protein, and DNA **[4]**.

Nitric oxide (NO) is a biologically active substance which is produced from L-arginine via a Ca^{2+} - dependent constitutive NO synthase (cNOS) or a Ca^{2+} - independent inducible NO synthase (iNOS) **[5]**, both NO synthases have been detected in gastric mucosal tissues of rats **[6,7]**. It has been widely accepted that in the digestive system, NO produced by cNOS is cytoprotective, while excessive NO produced by iNOS is cytotoxic **[8,9]**. It has also been reported that indomethacin induced ulceration in rats is associated with changes in NOS activity and hence NO production in the gastric mucosa **[10,11]**.

In addition it has been revealed that indomethacin-induced gastric mucosal injury in rats occurs

via gastric epithelial apoptosis [1] through enhanced expression of apoptosis-and inflammation-related genes in the gastric epithelial cells exposed to indomethacin [12,13].

L-Carnitine is a natural substance that acts as a carrier for fatty acids across the inner mitochondrial membrane for subsequent beta-oxidation [14,15]. By this mechanism L- carnitine profoundly influences fatty acid oxidation, and maintains low pools of fatty acid acyl-coenzyme A compounds, which are potentially toxic. It is well known also that L-carnitine and its acyl derivatives have antioxidant properties because of their inhibiting effect on xanthine oxidase activity [16], scavenging effect on ROS and suppression of hydroxyl radical production by the Fenton reaction, probably by chelating the iron required for the generation of hydroxyl radicals [17]. L-carnitine also inhibits neutrophil infiltration in ischemia reperfusion injury [18,19] and alter apoptosis, in skeletal muscle in rats with heart failure through inhibiting caspases, decreasing the levels of TNF-a as well as the number of apoptotic myonuclei [20].

L-carnitine and its acyl derivatives has been shown to be effective in various pathologic conditions characterized by increased oxidative stress such as cold restraint stress-induced gastric mucosal injury [21], ischemia-reperfusion injury during kidney transplantation in rats [19], renal failure [22], ischemia reperfusion coronary heart disease [17], and gentamicin induced toxicity that is primarily mediated via oxidative stress [23].

Thus through its antioxidant effect, alteration of NO, inhibition of neutrophil infiltration and inhibition of apoptosis, L-carnitine might have a relevant therapeutic outcome on indomethacin-induced gastric ulceration, the pathogenesis of which involves all of these changes. The purpose of the present study was to investigate this issue and to shed some light on the possible mechanisms underlying this gastroprotective effect.

Materials and Methods

Drugs and chemicals:

L-carnitine was obtained from Mepaco Co., Egypt, indomethacin from Pharco Pharmaceuticals, Egypt, and ranitidine hydrochloride from Glaxo-SmithKline-Beecham company, Egypt. NG-nitro-L-arginine methyl ester (L-NAME) was purchased from Fluka chemical company, USA Co. and Tween 80 from Merk, Germany. Other chemicals and organic solvents were of Analar grade.

All drugs were freshly prepared and suspended or dissolved in 1% Tween 80.

Animals:

Adult male albino rats each weighing 170-210 g were used in this investigation. They were purchased from the National Institute of Ophthalmology, Cairo, Egypt and kept to accommodate under standard animal house conditions (Faculty of Pharmacy, Cairo University), where they were provided with standard pellet diet and tap water ad libitum for at least 1 week before assignment to an experimental protocol. Rats were fasted 24 h prior to experiments but allowed free access to water except for the last hour before the experiment. The study was conducted in accordance with ethical procedures and policies approved by Animal Care and Use Committee of Faculty of Pharmacy, Cairo University.

Induction of gastric ulcers and drug treatments:

Two sets of experiments were performed. In the first set animals were randomly allocated into five groups (eight animals each): The first group received 1% Tween 80 and served as the normal control group. The second group received a single oral dose of indomethacin 30 mg/kg and served as ulcer control. The dose used in the present study is within the range reported in the literature to produce acute lesions in the rat gastric mucosa [11, 24-26]. The third, fourth, and fifth groups were pretreated 1 h before ulcer induction with 50 mg/kg po ranitidine hydrochloride, 100 and 300 mg/kg po L-carnitine respectively. The doses of L-carnitine used in this study was in homogeny with those reported in the literature [27,28] as well as the dose of ranitidine [10,26,29].

Animals in the second set were also randomly assigned into five groups (eight animals each). The first and second groups were treated as in the first set. The third group received L-NAME 50 mg/kg ip 1/2 h before indomethacin administration. The fourth and fifth groups received L-carnitine 300 mg/kg po 1 h before ulcer induction with indomethacin. The fifth group received in addition L-NAME 50 mg/kg ip 1/2 hour before L-carnitine.

Assessment of gross mucosal damage:

Animals were sacrificed 4 h after indomethacin administration and their stomachs were removed, opened along the greater curvature, rinsed with saline and pinned flat on a cork-board for lesion assessment. By the aid of a hand lens gastric mucosal injury was assessed by measuring the ulcer index (UI), which was expressed as the sum of ulcer lengths per stomach in mm [9].

Biochemical determinations:

Immediately after gross lesion examination, the gastric mucosa of each rat was scrubbed off and disrupted in ice cold saline using a homogenizer (IKA T 10, Germany) to prepare a 10% w/v homogenate. Aliquots of gastric mucosal homogenates were used for the estimation of lipid peroxides, reduced glutathione (GSH), myeloperoxidase (MPO) activity, and nitric oxide.

Determination of lipid peroxides:

One of the most frequently used biomarkers providing an indication of the lipid peroxidation level is the concentration of malondialdehyde (MDA), one of several byproducts of lipid peroxidation processes [30]. Gastric mucosal MDA was quantified by the thiobarbituric acid (TBA) test. Thiobarbituric acid reactive substances (TBARS) formed in tissue primarily consist of MDA, which form a pink or red adduct with 2 molecules of TBA (MDA-TBA_2) in acidic medium at high temperature. The pink adduct was extracted in n-butanol and measured spectrophotometrically at 535 and 520 nm. The difference in absorbance between the two readings was taken as the level of TBARS in the sample and was expressed as nmol/g tissue [31].

Determination of GSH:

Gastric mucosal GSH (mg/g tissue) was determined spectrophotometrically at 412 nm, after precipitation of protein in tissue homogenate with sulfosalicylic acid, using Ellman's reagent [32].

Determination of MPO activity:

Gastric mucosal MPO activity (U/g tissue) was measured according to the method of Bradley et al. [33]. Since MPO is located within the granules of neutrophils, extraction depends upon disrupting the granules with sonication and freeze-thawing 3 times in hexadecyltrimethylammonium bromide (HTAB) which acts as a solubilizer for the enzyme. The extracted enzyme was assayed with a dianisidine-H_2O_2 assay spectrophotometrically where the change in absorbance at 460 nm was measured at 1 min intervals.

Determination of NO:

Most of the biologically produced NO is oxidized to nitrate (NO_3) and nitrite (NO_2), totally designated as NOx. Determination of NOx in gastric mucosa was carried out according to the method of Miranda et al. [34] after deproteinization of the sample with absolute alcohol. The assay is based on reduction of any nitrate to nitrite by vanadium chloride (VCl_3). The total nitrite (intrinsic + nitrite obtained from VCl_3 reduction of nitrate) is then detected colourimetrically as an azo dye product of the Griess reaction at 540 nm and was expressed as nmol/g tissue.

Statistical analysis:

Data in graphs and tables are presented as mean values of 8 animals ± SEM. Comparison between the mean values of different groups was carried out by using one way analysis of variance (ANOVA), followed by Tukey-Kramer post hoc test for multiple comparisons. In all data analysis $p \leq 0.05$ was considered significant.

Results

Gastroprotective effect of l-carnitine on indomethacin induced gastric ulceration:

The gastroprotective effect of ranitidine and L-carnitine at 100 mg/kg and 300 mg/kg on indomethacin induced gastric damage was determined macroscopically through determination of the ulcer index. Indomethacin produced a remarkable high ulcer index. Pretreatment with ranitidine and the 2 doses of L-carnitine had significantly reduced the ulcerogenic effect of indomethacin by about 59%, 35%, and 53% respectively, with no significant difference between the gastroprotective effect of ranitidine and the higher dose of L-carnitine (Fig. 1).

Redox state of the gastric mucosa:

Indomethacin enhanced lipid peroxidation which is reflected by an almost three folds increase in gastric mucosal MDA concentration. Pretreatment with ranitidine and L-carnitine significantly reduced the elevated MDA concentration by about 40% for ranitidine and 54% for the lower dose of L-carnitine, while the 300 mg/kg, po L-carnitine almost normalizes the lipid peroxidation state (Fig. 2).

The gastric content of the antioxidant defense molecule GSH was lowered significantly by almost 40% after indomethacin administration compared to the control group. Pretreatment with ranitidine and L-carnitine significantly reverses this effect, where ranitidine increases GSH by 48% and the two doses of L-carnitine by 55% and 63% respectively (Fig. 3).

MPO activity:

Indomethacin significantly elevated MPO activity by 370% of the normal control value indicating a substantial neutrophil infiltration into the mucosa in response to injury. This was attenuated by ranitidine and the two tested doses of L-carnitine as evidenced by a significant reduction in gastric

mucosal MPO activity by about 47%, 30%, and 39% for ranitidine and the 2 doses of L-carnitine respectively. There was no significant difference between ranitidine and the higher dose of L-carnitine (Fig. 4).

Gastric mucosal nitrite content:

Indomethacin significantly reduced gastric mucosal nitrite level by about 44% compared to the control value. Pretreatment with ranitidine or L-carnitine almost restored the nitrite level to the normal control value (Fig. 5).

Effect of L-NAME:

Treatment with L-NAME (50 mg/kg, ip) significantly exacerbated gastric lesions, where it increased the ulcer index by about 55% as compared to ulceration induced by indomethacin alone. Addition of L-NAME to L-carnitine significantly reduced its gastroprotective effect which is reflected by a 60% increase in ulcer index compared to L-carnitine alone. However, in spite of this increase in ulcer index it was still 30% lower than that induced by indomethacin (Fig. 6).

L-NAME did not significantly affect gastric mucosal MDA or GSH concentrations as compared to the group treated with indomethacin alone. Similarly the addition of L-NAME to L-carnitine did not significantly alter the effect of the latter on these redox state parameters (Figs. 7,8).

L-NAME significantly reduced gastric mucosal nitrite concentration by about 27% compared to the indomethacin group, while the addition of L-NAME to L-carnitine reduced it by about 53% compared to the group given L-carnitine (Fig. 9).

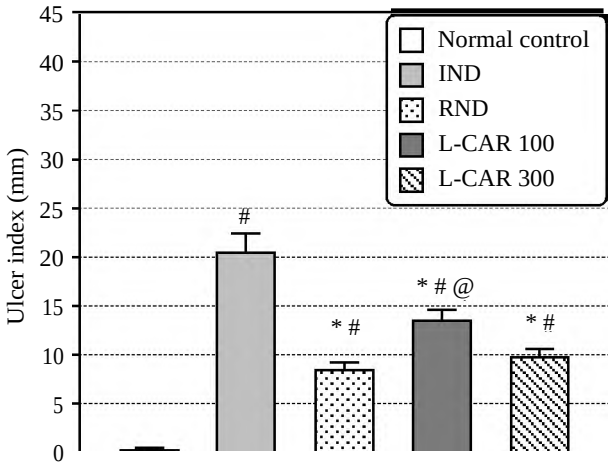

Fig. (1): Effect of ranitidine and L-carnitine on gastric lesions induced by indomethacin in rats. Values represent the mean ± SEM of 8 rats. * Significantly different from indomethacin at $p \leq 0.05$. # Significantly different from the control group at $p \leq 0.05$. @ Significantly different from ranitidine at $p \leq 0.05$.

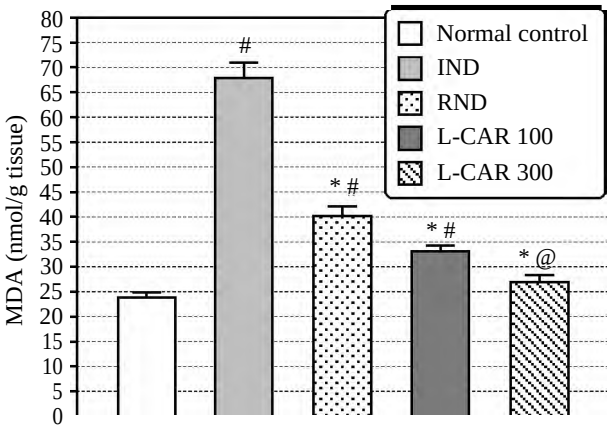

Fig. (2): Effect of ranitidine and L-carnitine on gastric mucosal MDA concentration in indomethacin-treated rats. Values represent the mean ± SEM of 8 rats. * Significantly different from indomethacin at $p \leq 0.05$. # Significantly different from the control group at $p \leq 0.05$. @ Significantly different from ranitidine at $p \leq 0.05$.

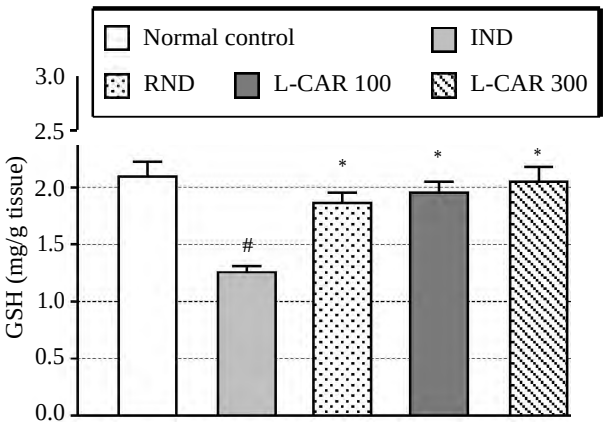

Fig. (3): Effect of ranitidine and L-carnitine on gastric mucosal GSH concentration in indomethacin-treated rats. Values represent the mean ± SEM of 8 rats. * Significantly different from indomethacin at $p \leq 0.05$. # Significantly different from the control at $p \leq 0.05$.

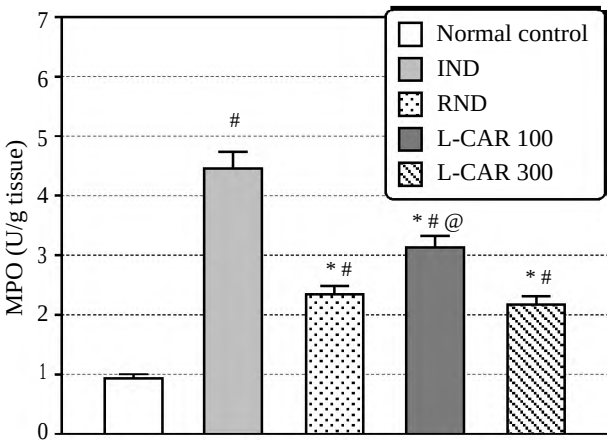

Fig. (4): Effect of ranitidine and L-carnitine on gastric mucosal MOP activity in indomethacin-treated rats. Values represent the mean ± SEM of 8 rats. * Significantly different from indomethacin at $p \leq 0.05$. # Significantly different from the control group at $p \leq 0.05$. @ Significantly different from ranitidine at $p \leq 0.05$.

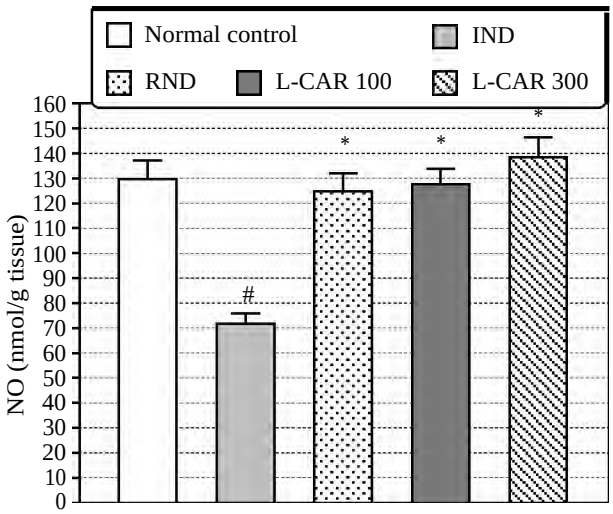

Fig. (5): Effect of ranitidine and L-carnitine on gastric mucosal nitrite concentration in indomethacin-treated rats. Values represent the mean ± SEM of 8 rats. * Significantly different from indomethacin at $p \leq 0.05$. # Significantly different from the control group $p \leq 0.05$.

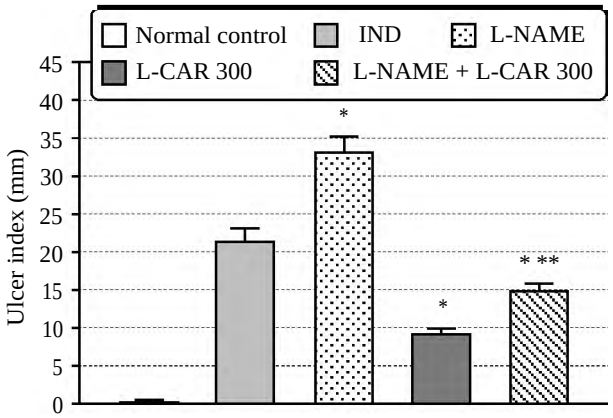

Fig. (6): Effect of L-NAME and L-carnitine on gastric lesions induced by indomethacin in rats. Values represent the mean ± SEM of 8 rats. * Significantly different from indomethacin at $p \leq 0.05$. ** Significantly different from L-carnitine at $p \leq 0.05$.

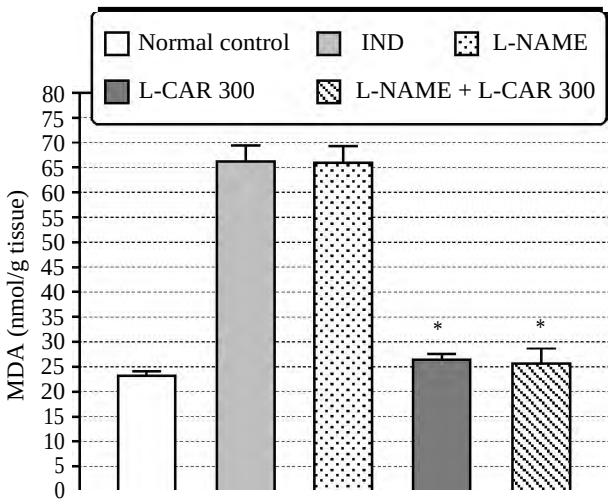

Fig. (7): Effect of L-NAME and L-carnitine on gastric mucosal MDA concentration in indomethacin-treated rats. Values represent the mean ± SEM of 8 rats. * Significantly different from indomethacin at $p \leq 0.05$.

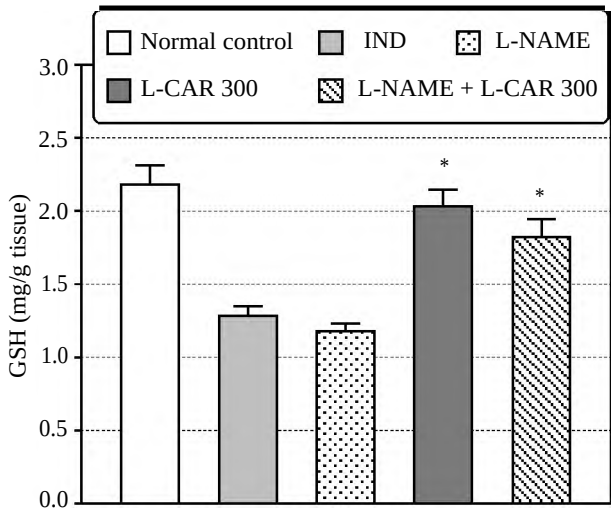

Fig. (8): Effect of L-NAME and L-carnitine on gastric mucosal GSH concentration in indomethacin-treated rats. Values represent the mean ± SEM of 8 rats. * Significantly different from indomethacin at $p \leq 0.05$.

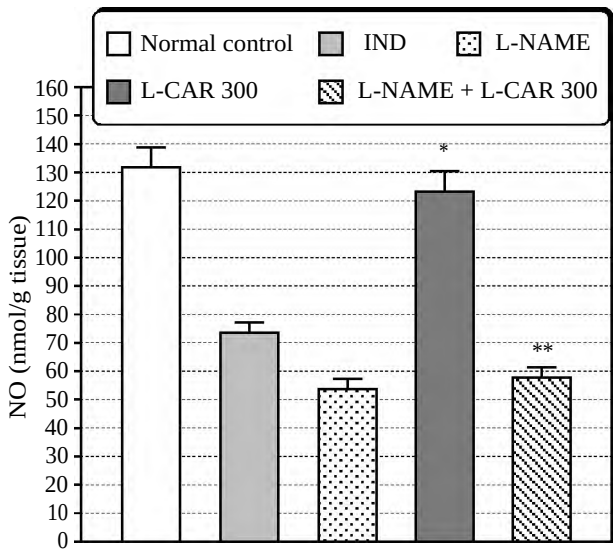

Fig. (9): Effect of L-NAME and L-carnitine on gastric mucosal nitrite concentration in indomethacin-treated rats. Values represent the mean ± SEM of 8 rats. * Significantly different from indomethacin at $p \leq 0.05$. ** Significantly different from L-carnitine at $p \leq 0.05$.

Discussion

It was reported earlier in a preliminary study that L-carnitine decreased indomethacin-induced gastric mucosal injury **[28]**, prevented the stress-induced increase in gastric lesion index **[35]**, and inhibited ethanol-induced gastric mucosal injury in rats **[36]**. However the mechanisms underlying the gastroprotective effect of L-carnitine was not fully investigated. The present study examined the anti-ulcer effect of L-carnitine compared to ranitidine as a standard anti-ulcer agent and the involvement of its antioxidant activity and the possible

role of nitric oxide in the mechanisms responsible for this anti-ulcer activity.

Despite the fact that many factors contribute to the gastrotoxicity of NSAIDs as non-selective inhibition of cyclooxygenase and consequent reduction in prostaglandins synthesis [2,3], yet ROS play a crucial role in indomethacin and other NSAIDs induced gastropathy [24,37-40]. Thus any agent that can prevent this oxygen free radical damage should serve as a mucosal shield.

The present study demonstrated that indomethacin administration induced severe gastric lesions associated with derangement in the redox state of the gastric mucosa as evidenced by a significant increase in MDA level which was in agreement with other previous studies [24,39,41]. Indomethacin increases the generation of hydroxyl radicals and superoxide anions [42] which initiate lipid peroxidation and at the same time oxidizes GSH resulting in significant reduction of its gastric concentration [43,44]. Ranitidine and L-carnitine reduced the ulcer index significantly in a dose dependent manner and the effect of the higher dose of L-carnitine was comparable to ranitidine which was used as a standard anti-ulcer agent. L-carnitine and ranitidine improved the antioxidant status through reduction of MDA, which was normalized by the higher dose of L-carnitine, and elevation of GSH which closely paralleled the reduction in gastric mucosal injury suggesting that lipid peroxidation and reduced GSH plays a significant role in the pathogenesis of the gastric mucosal injury induced by indomethacin as noted above. This was confirmed in other studies which demonstrated that treatment with superoxide dismutase (SOD; a superoxide anion scavenger) and dimethyl sulfoxide (DMSO; a hydroxyl radical scavenger) inhibited the increase in vascular permeability and mucosal injury induced by indomethacin [37]. L-carnitine induced reduction in MDA in this study may be due to its well known antioxidant effect since L-carnitine and its acyl derivatives inhibit xanthine oxidase activity [16], scavenge ROS and suppress hydroxyl radical production by the Fenton reaction, probably by chelating the iron required for the generation of hydroxyl radicals [17]. L-carnitine also enhances the transport of fatty acids into mitochondria for energy production and inhibits the microsomal peroxidation [45]. The increase in GSH may be due to the exertion of thiol and methionine sparing activity by L-carnitine [46] and may also be due to increased NADPH generation through increased fatty acid metabolism [27].

The increase in gastric mucosal MPO activity in indomethacin treated rats is an indication of the injurious state as a result of neutrophil infiltration. This is in accordance with the work of Motawi et al. and Dengiz et al. [11,44]. Leukocytes might contribute to ulceration by occluding microvessels, thereby reducing mucosal blood flow, and by acting as a potential source of ROS since they contain NADPH oxidase that reduces molecular oxygen to superoxide anion radical, resulting in its conversion to H_2O_2 [47,48]. The enhanced neutrophil infiltration might be attributed to the reported increase in TNF-a and leukotriene synthesis in indomethacin induced gastric injury and these inflammatory mediators stimulate neutrophil adherence by upregulation of adhesion molecules [49]. Ranitidine and L-carnitine significantly attenuated neutrophil infiltration as evidenced by the remarkable reduction of MPO activity thus reducing ROS produced from the recruited and activated leukocytes. This inhibitory effect on neutrophil infiltration might be due to L-carnitine induced decrease in TNF-a [20] thus inhibiting neutrophil adherence [47]. This is in accordance with the work of Derin et al. who demonstrated that L-carnitine reduced MPO and exerted a gastroprotective effect against gastric mucosal lesions due to leukocyte infiltration induced by ischemia-reperfusion [18].

Indomethacin severely reduced gastric mucosal nitrite content reflecting a decrease in NOS activity and this was confirmed in previous studies [10,50]. On the other hand several investigators demonstrated that indomethacin increased the expression of iNOS in gastric epithelial cells [51,52]. However the overall effect was a reduction in gastric mucosal NO content which might be due to indomethacin induced upregulation of endothelin-1 that suppresses gastric mucosal constitutive nitric oxide synthase (cNOS) [25]. Gastric mucosal nitrite concentration was preserved after pretreatment with L-carnitine. This was associated with a decrease in indomethacin-induced gastric mucosal injury. This suggests that the mechanism of the gastroprotective effect of L-carnitine was at least partly mediated by NO. The mechanisms through which nitric oxide prevents mucosal damage involve increased gastric blood flow, reduced neutrophil adhesion, and increased mucus secretion [53].

This normalization in gastric mucosal nitrite content by L-carnitine could be attributed to a reduction in ROS formation resulting in a higher NO availability. However, it is also possible that the reduction in ROS by L-carnitine increases the enzymatic production of NO from cNOS, due to a decreased oxidative uncoupling of the enzyme

[54] . Koeck and Kremser have shown that ROS generation is involved in the decrease of NOS activity or the decreased potency of endothelial NOS activation by added Ca^{2+} in human skin fibroblasts **[55]** . A third possibility is that L-carnitine could increase the cytoprotective cNOS, where some authors have shown that L-carnitine increased expression of cNOS in human endothelial cell culture **[56]** and increased NO in fructose fed hypertensive rats **[57]** and in tumor tissue in mice **[58]** .

To investigate the role of NOS in the gastroprotective effect of L-carnitine pretreatment with L-NAME, a non-selective NOS inhibitor, has been carried out. Administration of L-NAME with indomethacin enhanced gastric mucosal damage. This confirmed that NO serves as a mediator of gastric mucosal integrity, protecting against indomethacin injury **[50,59-61]** . When combined with L-carnitine, L-NAME reduced the gastroprotective effect of L-carnitine by one half and attenuated its effect on gastric mucosal nitrite content, without altering the levels of MDA or GSH. This means that inhibiting NOS especially cNOS, since the other isoform iNOS is cytotoxic in the gastrointestinal system **[8,9]** deprived L-carnitine of part of its gastroprotective effect and at the same time reduced gastric mucosal nitrite content indicating that L-carnitine could probably protect cNOS from oxidative uncoupling. The correlation between the reduction in nitrite content and the gastroprotective effect of L-carnitine when given with L-NAME was not high. The reduction in nitrite content was almost to the same level as that attained by indomethacin alone but the gastroprotective effect was reduced to a lesser extent and the ulcer index is still significantly lower than that of indomethacin. This means that although L-NAME abolished L-carnitine potentiating effect on NOx yet part of the gastroprotective effect is retained which means that possibly other mechanisms apart from increased NO availability or production, could participate in the overall gastroprotective effect of L-carnitine in indomethacin induced ulcer. These other mechanisms include the antioxidant activity and reduction of ROS, inhibition of neutrophil infiltration as proved in this study. Other possible mechanisms may include alteration of apoptosis and inhibition of caspases **[20]** , since indomethacin-induced gastric mucosal injury in rats occurs via gastric epithelial apoptosis **[1]** but this requires further work to clarify it.

In conclusion, L-carnitine has a dose-dependent gastroprotective effect against indomethacin induced ulcer which is comparable to the H_2 antagonist ranitidine. This gastroprotective effect is probably due to several mechanisms that include antioxidant activity, inhibition of leukocyte infiltration in the gastric lesion, increasing the availability of NO in the gastric mucosa through reduction of ROS and increasing the enzymatic production of NO from cNOS, probably due to decreased oxidative uncoupling of the enzyme.

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