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The ameliorating role of L-arginine and L-citrulline on gastric mucosal damage and lipid peroxidation in adult female rabbits with gastric ulceration induced by indomethacin

Bassim Khamess Kuoti Al-Rekabi*

University of Sumer, College of Agriculture, Department of Animal Production, Thi-Qar, Iraq

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ABSTRACT

This study was designed to evaluate the anti-ulcerogenic effect of L-Arginine and L-Citrulline and their combination on indomethacin-induced gastric mucosal damage in adult female rabbits. Thirty adult female rabbits 1.25-1.75 kg, 24-26 weeks old were randomly divided into five equal groups, 6 animals each as in following: Control group: received distilled water orally 5 ml/kg. First treated group (T1): received indomethacin orally 50 mg/kg/day for 48 hours. Second treated group (T2): received indomethacin for 48 hours and after 30 minutes received L-Arginine orally 2.5 mmol/kg/day for 7 days. Third treated group (T3): received indomethacin for 48 hours and after 30 minutes received L-Citrulline orally 2.5 mmol/kg/day for 7 days. Fourth treated group (T4): received indomethacin for 48 hours and after 30 minutes received a combination of half a dose of L-Arginine 1.25 mmol/kg/day and L-Citrulline 1.25 mmol/kg/day for 7 days. The animals were sacrificed after lasted 10 hours. The obtained results illustrated that female rabbits treated with L-Arginine and L-Citrulline significantly prevented gastric ulcer induction through significant ($P<0.05$) reduced in gastric juice volume and ulcer area with 100% inhibition of ulceration, significant ($P< 0.05$) increase in gastric pH and almost return to its normal level compared to control value. It has been concluded that combination of L-Arginine and L-Citrulline appeared the best protective effect against gastric ulcer better than each drug alone in the indomethacin-treated group with a normal histopathological structure related with normal glandular stomach, numerous number of parietal cells in lamina propria.

* Corresponding Author

Name: Dr. Bassim Khamess Kuoti Al-Rekabi
Phone: +9647804321518
Email: kh_bassim@yahoo.com

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INTRODUCTION

Gastric ulcer is a widespread gastrointestinal erosion. There are many endogenous and exogenous mediators are related to producing gastric ulceration, for instance, excessive secretion of gastric acid, utilizations of non-steroidal anti-inflammatory drugs, stress and huge consumption of tobacco, alcohol and caffeine, psychological stress, pepsin and stomach juice, fatty foods and Helicobacter pylori infection-triggered tissue necrosis, and a number of defense mechanisms of gastrointestinal mucosa are local blood flow, mucus/bicarbonate secretion, and cellular growth (Onasanwo *el al.,* 2001). Indomethacin is one of the most widely used NSAIDs. It causes ulceration of gastrointestinal mucosa by irritant actions causing alterations in mucosal permeability or suppression in synthesis of prostaglandin (PGs) that resulting from inhibition of cyclooxygenase (COX), a key enzyme in biosynthesis of PGs which play an essential

protective role in stomach by stimulating synthesis and secretion of mucus and bicarbonate, increasing mucosal blood flow and promoting epithelial proliferation (Heeba *et al.,* 2009). Therefore, gastric ulcer induced by the production of free radical, induce lipid peroxidation, inhibition of prostaglandins and leukocyte infiltration (Kwiecien *et al.,* 2014). L-arginine is a biologic precursor of nitric oxide (NO) synthesis, an endogenous messenger molecule involved in a variety of endothelium-dependent physiological effects in the cardiovascular system by its effects on endothelial resulting relaxing factor **(**Wu and Meininger, 2000). It has serious significance in protection of normal blood pressure (Umans and Levi, 1995), myocardial function **(**Hare and Colucci,1995), inflammatory response (Wileman *et al.,* 1995), apoptosis (Brune *et al.,* 1995), and protection against oxidative damage **(**Wink *et al.,* 1995). L-arginine is one of the semi-essential amino acids, it is found in most mammals and play a vital biological function for the body (Raber *et al.,* 2012). It acts as mediators of several important functions of the body including an increase of immunity, enhances cell division, promotes wound healing and modified secretion of essential hormones (Walker *et al.,* 2001). It can be produced from citrulline metabolism by the action of argininosuccinate synthetase and argininosuccinate lyase and catabolized by arginase (Yu *et al.,* 1997), citrulline is synthesized from glutamine, glutamate and proline in the mitochondria of enterocytes, released from the small intestine, and taken up primarily by kidneys for L-arginine production. The nitric oxide (NO) is synthesized from L-arginine by nitric oxide synthases, through the L-arginine-nitric oxide pathway (Pedrycz and Siermontowski, 1997). NO synthase (NOs) is metabolized L-arginine to L-citrulline and NO (Michel and Feron, 1997). The purpose of this study to investigate the strength potential anti-ulcerogenic in both L-arginine and L-Citrulline, by making a sort of a combination between both compounds above, the results would be compared with L-arginine, and L-Citrulline gave orally to indomethacin-induced gastric mucosal damage in adult female rabbits.

MATERIALS AND METHODS

Administration and Preparation

L-Arginine (L-ARG) and L-Citrulline (L-CIT) obtained from (Sigma, St. Louis, MO, USA) was dissolved in 2 ml distilled water and prepared freshly each time and given at similar doses (2.5 mmol/kg/day) and administered orally by gastric gavages as a single dose after 30 minutes later for indomethacin prior to induced gastric ulcer, indomethacin 50 mg/kg obtained from (KGP. for chemical industries, USA) was dissolved in 2 ml distilled water and given orally. Amprolium HCl (Joprol 60%, 600 mg) obtained from (Vet. and AGR. Med. IND. COM, Jordan) and given orally. Ivermectin injection I.P, Mectinex 50 ml obtained from (Stanex drugs and chemical PVT. LTD, India) and given intraperitoneally. The animals were sacrificed after 10 hours later, and gastric content with the stomach wall was collected. Control received 5 ml/kg of distilled water by oral route administration.

Experimental Animals

A total of thirty healthy adult female domestic rabbits (*Lepus cuniculus*) with body weight ranged 1.25-1.75 kg, 24-26 weeks were brought from the local markets in Basrah province. Rabbits were kept for an adaptation period for 1 month in the animal house of College of the Veterinary Medicine/University of Basrah. The experimental animals were kept in individual cages, provided with pellet foods and tap water *ad libitum*. The animals were given anticoccidosis drug (Amprolium) at a dose of 0.5 ml/L and ivermectin at a dose of 0.1 mg/rabbit for control of internal and external parasites through drinking water daily for 2 weeks. These animals are maintained in air-conditioned quarters 24°C under standard husbandry condition with alternate 12 hours' light /dark by use of two fluorescent lamps, and the humidity rate was about 50 %.

Experimental Design and Study Strategy

After the acclimatization period, animals were fasted for 24 hours before gastric ulcer induction, although allowed free admission to water excluding for last hour before induction of gastric ulceration. Gastric ulcer generated by indomethacin according to the method described by (Elegbe and Bamgbose, 1976). The animals were randomly divided into five equal groups, 6 animals each as in the following: Control group: received orally distilled water 5 ml/kg. First treated group: received indomethacin orally 50 mg/kg/day for 48 hours. Second treated group (T2): received indomethacin for 48 hours and after 30 minutes received L-Arginine orally 2.5 mmol/kg/day for 7 days. Third treated group (T3): received indomethacin and after 30 minutes received L-Citrulline orally 2.5 mmol/kg/day for 7 days. Fourth treated group (T4): received indomethacin and after 30 minutes received a combination of a half dose of L-Arginine 1.25 mmol/kg/day and L-Citrulline 1.25 mmol/kg/day for 7 days.

Induction of Gastric Ulcer

Gastric ulcer was induced in adult female rabbits by giving indomethacin (KGP. for chemical industries, USA) orally by one ml size syringe at a dose 50 mg\kg suspended in 2 ml of D.W for 48 hours. After 48 hours of each experimental period, blood samples were taken by cardiac punctures then the

animals were scarified. A midline abdominal incision was performed. The stomachs ligated from esophageal and pyloric opening, rapidly removed, opened along their greater curvature spread on a paraffin plate and gently washed with normal saline. The gastric juice was collected in the test tube. Parts of gastric mucosa were removed for other laboratory tests. Finally, the stomach was immersed in 10% formalin for histopathological examination according to a method described by (Alphin and Ward,1967).

Determination of Gastric Juice Volume

Gastric juice collected from each stomach was centrifuged at 3000 rpm for 10 minutes to remove any solid debris, gastric volume (ml) of the supernatant was measured by graduated cylinder according to a method described (Almayah, 2012).

Determination of Gastric pH

Acidity degree (pH) of gastric juice from animal stomach was determined by using pH meter apparatus (HI 9021)

Determination of Inhibition in Gastric Ulceration (%)

Inhibition gastric ulceration (I.G.U %) was calculated according to the following equation

$$\text{I. G. U \%} = \frac{\textit{ulcer inhibition of control} - \textit{ulcer inhibition of treated}}{\textit{ulcers Index of Control}} \times 100$$

Measurement of Gastric Tissue MDA

Calculation of Malondialdehyde

One ml of 10% trichloroacetic acid (TCA) was added for each one mg of ulcerated gastric tissue in a manual tissue homogenizer. Homogenization was performed for 15 minutes. Centrifugation at 3000 rpm for 10 minutes and supernatant was obtained. An equal volume of 0.06% of thiobarbituric acid substance (TBAS) was added to the supernatant. Heating for 20 minutes at 100°C by using a water path. Reading by spectrophotometer at 532 nm according to the method described by (Cassini *et al.,* 1986).

Statistical Analysis

In this study, ANOVA Analysis and LSD tests are used according to (IBM SPSS, version 20) program at (P≤0.05) to find the means for all treatments (IBM SPSS, 2011).

RESULTS

The results of a current study on a table (1) demonstrate that female rabbits received orally indomethacin (50 mg/kg) for 48 hours caused significant increase (P≤0.05) in gastric juice volume, ulcer area, gastric tissue MDA, significant decrease (P≤0.05) in gastric pH compared to control group. Whereas female rabbits treated with L-ARG, L-CIT and their combination showed significant reduced (P<0.05) in gastric juice volume, ulcer area, 100% inhibition in gastric ulceration and gastric tissue MDA, a significant increase (P≤0.05) in gastric pH compared to groups treated with Indomethacin. It is also clear from table (1) that combination of L-ARG 1.25 mmol/kg/day and L-CIT 1.25 mmol/kg/day caused highly significant declined in gastric juice volume, ulcer area and gastric tissue MDA with 100% inhibition in gastric ulceration, and highly significant (P< 0.05) increase in gastric pH and almost return to its normal level compared to control.

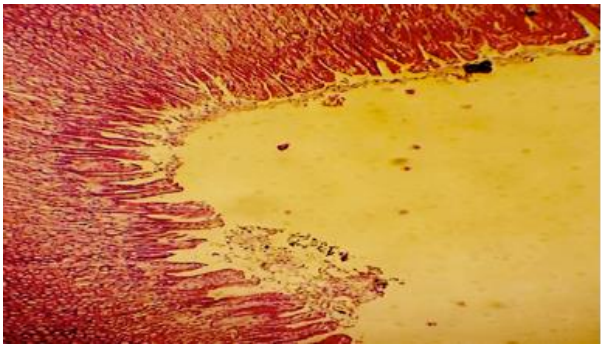

Figure 1: Stomach normal control of female rabbits revealed normal gastric mucosa, stomach lamina properia with parietal cells — stain (H&E) 100X.

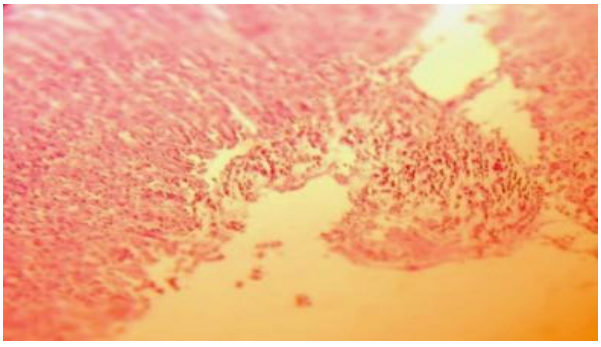

Figure 2: Stomach of female rabbits treated with Indomethacin for 48 hours induced gastric ulcer revealed stomach lamina propria with parietal cells in the prominent cells with interstitial edema. Stain (H&E) 100X.

Histopathological Examination of Gastric Mucosal Layer

The results of histopathological changes revealed that gastric mucosal layer of normal control of female rabbits showed normal gastric mucosa, gastric lamina propria with parietal cells as shown in figure (1), while gastric mucosal layer of female rabbits treated with indomethacin showed histopathological changes include gastric lamina propria with parietal cells in prominent cells with interstitial oedema. In addition, ulceration with glandular proliferation of reactive remaining mucosa,

fibrosis and cartilage as shown in figure (2), but gastric mucosa of female rabbits treated with L-ARG at a dose 2.5 mmol/kg/day and L-CIT at a dose 2.5 mmol/kg/day and their combination for 7 days of treatment revealed normal glandular stomach, numerous number of parietal cells in lumina propria as shown in figures (3), (4) and (5).

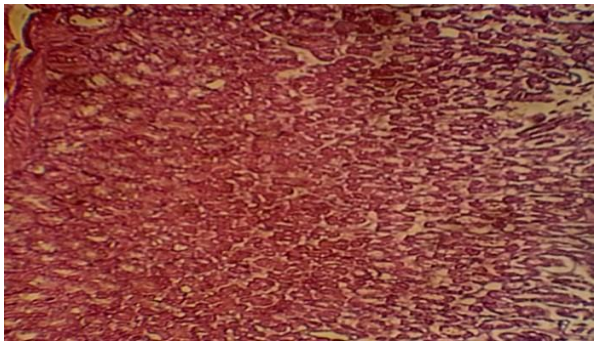

Figure 3: Stomach of female rabbit gastric ulceration treated with L-ARG at a dose 2.5 mmol/kg/day for 7 days of treatment revealed normal glandular stomach and many numbers of parietal cells in lamina propria. stain (H&E) 400X

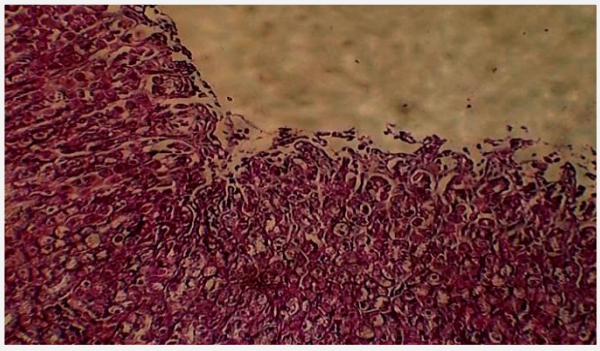

Figure 4: stomach of female rabbit gastric ulceration treated with L-CIT at a dose 2. 5 mmol/kg/day for 7 days of treatment revealed normal architecture and lamina propria filled with parietal cells. Stain (H&E) 400X.

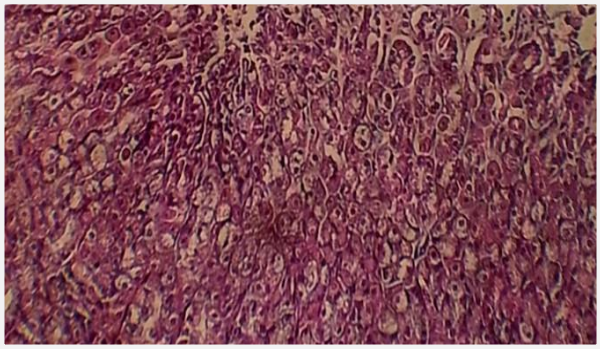

Figure 5: Stomach of female rabbit gastric ulceration treated with a combination of L-ARG at a dose 1.25 mmol/kg/day + L-CIT at a dose 1.25 mmol/kg/day for 7 days of treatment revealed normal glandular stomach, normal architecture and lamina propria filled with parietal cells. Stain (H&E) 400X.

DISCUSSION

According to (Goyal and Bhattacharya, 1999) who demonstrate that gastric mucosal damage initiates from variation between a destructive and distrustful factor of gastric mucosa. However, the composition of pepsin and gastric acid the offensive factors, whose proteolytic product is buffered by secretion of mucin, cell shedding, glycoprotein, cell proliferation and prostaglandins. In order to recover stability, diverse therapeutic agents are used to inhibit gastric acid secretion or to stimulate the mucosal defence mechanism by increasing the mucus production that protects the surface epithelial cells or interfering with the prostaglandins synthesis (Goyal and Sairam, 2002). As a result, stress factors induced ulcers are possibly mediated by histamine released with improvement in the acid secretion, a decrease in produced mucus and generation of free radicals etc., mast cell activation, changes in prostaglandin generation, cytokine release and break of the normal cytoprotective mechanism (Sharma *et al.,* 2012). The mechanism of pathogenesis in indomethacin induced gastric ulcer is characterized by gastric acid hypersecretion, mucosal resistance declination and gastric emptying advertising. Indomethacin inhibits prostaglandin biosynthesis coupled with free radical's generation (Ajani *et al.,* 2014). Indomethacin-induced gastric mucosal damage which is confirmed by grossly and histopathological examination. The mortality rate takes place may be as a result of gastric damage and bleeding or may be hepatotoxic results of indomethacin (Abatan *et al.,* 2006). It is clear from obtained results that oral administration of indomethacin (50 mg/kg) for 48 hours caused significantly decreased in gastric pH, an increase in gastric juice volume, gastric ulcer and gastric tissue MDA. The gastric ulcer caused by indomethacin is chiefly attributed to various progressions include reactive oxygen species generation, initiation of lipid peroxidation, infiltration of leukocyte, initiation of apoptosis and inhibition of prostaglandin biosynthesis (Bech *et al.,* 2000). These results are in agreement with findings obtained by (Abdallah *et al.,* 2011), who reported that decrease in gastric pH might be attributed to decrease of mucin secretion which allows hydrogen ions and pepsin to be diffused into mucosa from the lumen. Reverse diffusion of acid and pepsin into tissue stimulate further secretion of acid and pepsin to cause more damage. Also, an indomethacin-induced significant increase in gastric tissue MDA. The significant increase in gastric juice volume after oral exposure to indomethacin may be attributed to either formation of free radicals or inhibition of prostaglandin biosynthesis which lead to increased gastric

Table 1: The protective role of L-Arginine and L-Citrulline and their combination on gastric juice volume, gastric PH, ulcer area, inhibition percentage, and gastric tissue MDA in female rabbits with gastric ulceration induced by Indomethacin

Parameters / Treatment	Gastric juice volume (ml)	Gastric pH	Ulcer area (mm)	Inhibition gastric ulcer (I.G.U) %	Gastric tissue MDA (nmol/mg)
Control D.W (5ml/ kg)	10.4 ± 2.30b	2.31±0.30a	0	-	3.48±1.67 b
Indomethacin (50 mg/kg)	21.06± 3.42a	1.38±0.22b	7.23± 2.35	-	12.28±2.67a
Indomethacin+L-ARG (2.5 mmol/kg/day)	10.45± 1.04b	2.53±0.28a	0	100%	2.67±1.19b
Indomethacin+L-CIT (2.5 mmol/kg/day)	10.83± 1.09b	2.68±0.41 a	0	100%	2.07±0.71 b
Indomethacin + L-ARG (1.25 mmol/kg/day +L-CIT 1.25 mmol/kg/day)	7.23 ± 1.03c	2.43 ± 0.12a	0	100%	2.80±1.07b
LSD	3.17	0.93	-	-	8.8

Small letters mean differences between groups at P≤0.05; (Mean ± SD)

acid secretion (Sabiua *et al.,* 2015). The gastric mucosal ulcer induced by indomethacin caused gastric oxidative stress that led to damage the lipid and stimulates lipid oxidation which led to an increase of MDA levels. This result is in agreement with results obtained by (Biplab *et al.,* 2011). Orally supplementation of amino acids L-arginine, L-citrulline and their combination for 7 days of treatment caused a significant increase in gastric pH, and decrease gastric juice volume, gastric ulcer and gastric tissue MDA. The increase in gastric pH confirmed the anti-secretory activity of L-arginine and L-citrulline (Usman *et al.,* 2014).

On the other hand, L-arginine and L-citrulline decrease gastric juice volume due to its antioxidant effect that lower secretion of gastric volume by acting on gastric mucosa, inhibiting the generation of reactive oxygen species that initiate oxidative stress in a gastric lumen (El-Saka *et al.,* 2014; Morita et al*.,* 2014). The number of gastric ulcer score decreases as a result of decrease gastric volume and increase gastric pH (Tsuboi *et al.,* 2018). These findings are in agreement with (Saad, 2013; Fu *et al.,* 2013). Moreover, L-arginine and L-citrulline caused a significant decrease in gastric tissue MDA. This is due to potent antioxidant properties, and free radicals scavenging and decrease lipid peroxidation (Ahmed *et al.,* 2015; Oluwole *et al.,* 2014). This result is in agreement with results obtained by (El-Kirsh *et al.,* 2011; Gou *et al.,* 2011). In addition, L-Arginine acts as a substrate for nitric oxide biosynthesis of a radical involved in different properties such as relaxation of smooth muscle and host defence (Graf *et al.,* 2001).

Furthermore, nitric oxide may be essential for the reliability of gastric mucosa both in the health and disease through its antimicrobial acting and by effecting mucous production by gastrointestinal mucosa. Nitric oxide also directly activates cyclooxygenase (COX) enzymes. NO activates COX by binding directly to the heme prosthetic group. Both COX-1 and COX-2 give gastric defence during the action of prostaglandins E and F2α. Prostaglandins acts via stimulation of epithelial cells to release mucus and bicarbonate inhibit gastric secretion, increase mucosal blood flow, down normalize the release of inflammatory mediators which supply generation of mucosal damage indefinite conditions and increase speed ulcer healing (Tsai *et al.,* 1994). L- Arginine and L- citrulline improved and significantly enhanced gastric mucosal damage together with increase mucus production and gastric pH content, inhibition of edema in addition to infiltration of neutrophil in the submucosa (Wallace, 2008). (Pedrycz and Siermontowski, 2017) Showed that L-citrulline could be voluntarily transformed to L-arginine in the kidney, vascular endothelium and other tissues, therefore, elevated plasma and tissue levels of L-Arginine.

Furthermore, L-arginine can slow down raise inducible nitric oxide synthase (iNOS) expression caused by myocardial ischemia-reperfusion. As a result, the inhibitory effect of L-citrulline on expression OF iNOS may be mediated through alteration into L-arginine (Zimmerman *et al.,* 1997). The elevation of mucus production contributes to defensive produce of amino acids. These influences possibly due to defensive factors activation concerned in defense of gastric mucosa and inhibition of offensive factors. (Suzuki *et al.,* 1998) Confirmed that decrease of neutrophil infiltration into gastric ulceration supports the preventive of gastric ulcers in rats. Neutrophils mediate lipid peroxidation through the production of superoxide anions (Hayashi *et al.,* 2005). Generation of free radicals from the infiltration of neutrophils in ulcerated

gastric tissues has an inhibitory effect on gastric ulcers in rats (Kehinde *et al.,* 2015).

CONCLUSION

The results of the current study proposed that L-Arginine and L-Citrulline have anti-ulcerogenic activity against indomethacin-induced gastric ulceration. Histology revealed relatively reduced gastric mucosal damage and inhibited edema.

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