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L-CARNITINE PROTECTS GASTRIC MUCOSA BY DECREASING ISCHEMIA-REPERFUSION INDUCED LIPID PEROXIDATION

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Studies have shown that reactive oxygen metabolites and lipid peroxidation play important roles in ischemia-reperfusion injury in many organs such as heart, brain and stomach. The aim of this study is to evaluate the antioxidant effect of L-carnitine on gastric mucosal barrier, lipid peroxidation and the activities of antioxidant enzymes in rat gastric mucosa subjected to ischemia-reperfusion injury. Rats were subjected to 30 min of ischemia followed by 60 min of reperfusion. L-carnitine (100 mg/kg), was given to rats intravenously five minutes before the ischemia. In our experiment, lesion index, thiobarbituric acid reactive substances, prostaglandin E₂ and mucus content in gastric tissue were measured. The results indicated that the lesion index and the formation of thiobarbituric acid reactive substances increased significantly with the ischemia-reperfusion injury in the gastric mucosa. L-carnitine treatment reduced these parameters to the values of sham operated rats. The tissue catalase and superoxide dismutase activities and prostaglandin E₂ production decreased significantly in the gastric mucosa of rats exposed to ischemia-reperfusion. L-carnitine pretreatment increased the tissue catalase activity and prostaglandin E₂ to the levels of sham-operated rats but did not change superoxide dismutase activity. There were no significant difference in glutathione peroxidase activity and mucus content between the groups in the gastric mucosa. In summary, L-carnitine pretreatment protected gastric mucosa from ischemia-reperfusion injury by its decreasing effect on lipid peroxidation and by preventing the decrease in prostaglandin E₂ content of gastric mucosa.

Key words: *Ischemia-reperfusion, lipid peroxidation, antioxidant enzymes, gastric lesion, L-carnitine*

INTRODUCTION

It has been reported that reactive oxygen metabolites (ROM) play important roles in the pathogenesis of inflammation, atherosclerosis, neurodegenerative diseases, diabetes mellitus, apoptosis and ischemia-reperfusion (I/R)(1-4). The role of ROM has been of great interest in I/R injury of heart, small intestine, liver, kidney and brain (1,5,6). Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) protect gastric mucosa against I/R injury by inhibiting the production of ROM by means of detoxifying superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) eventually (7-9).

The ischemia itself causes tissue damage and eventual death, but further injuries can occur while oxygen is reintroduced to the tissue. The possible factors that cause ischemia-reperfusion injury are: 1) the over- influx of calcium ions into the cells 2) the increase of the ROM production (5,6). The increase of calcium level in the cytosole causes various cell dysfunctions, such as impairment of oxidative phosphorylation in mitochondria which causes energy deficits, cytoskeletal damage, activation of phospholipase A_2 (PLA $_2$) and production of xanthine oxidase from xanthine dehydrogenase through protease activation. The activation of PLA $_2$ induces production of platelet activating factor and arachidonic acid from phospholipid of the plasma membrane. The degradation of phospholipid leads to the injury of the cell membrane and increases the permeability (10). During ischemia, ATP is catabolized to hypoxanthine and is accumulated within the tissue. With reperfusion, xanthine oxidase can utilize hypoxanthine and oxygen to form O_2^- and H_2O_2 (5,10). O_2^- and H_2O_2 may then interact to produce the highly reactive hydroxyl radical. Lipid peroxidation mediated by free radicals is believed to be one of the important causes of cell membrane destruction and cell damage, because the cell membrane contains much lipid, especially unsaturated fatty acid (4,5,11,12). It is well known that the gastric erosions due to free radicals have prevented by free radical scavengers or antioxidants (13-16). It is demonstrated that L-propionyl carnitine has a pronounced protective effect against ischemia-reperfusion damage (17). L-carnitine, a natural component of mammalian tissue, is a necessary factor in the utilization of long chain fatty acids to produce energy. Furthermore, carnitine improves the turnover of fatty acids peroxidated by the free oxygen radicals which were produced during normal metabolism (18). Reznick et al. have shown that L-carnitine suppressed hydroxyl radical production in the Fenton Reaction, probably by chelating the iron required for the generation of hydroxyl radicals (19). Furthermore, L-carnitine inhibited xanthine oxidase activity thus may act as a free radical scavenger and stabilizer of cell membranes (18,19). Antioxidant effects of L-carnitine have consequently been used for degenerative brain diseases, myocard and skeletal muscle ischemia (20,21).

It is not known whether L-carnitine has any effect on maintenance of mucosal integrity and lipid peroxidation activities of antioxidant enzymes. The aim of this

study is to evaluate the effect of L-carnitine on ischemia-reperfusion induced impairment of the gastric mucosal barrier. However, we examined the changes in TBARS concentrations and the activities of SOD, CAT and GPx in the gastric mucosa.

MATERIALS AND METHODS

Preparations of animals:

Thirty male Wistar rats, weighing about 200 g were housed at 22 ± 1 °C on a 12 h day-night cycle with a standard diet and water ad libitum. The study protocol was approved by the Akdeniz University Animal Care and Use Committee.

Three groups of animals were studied:

Group 1, sham-operated rats (n=10), 5 min before the beginning of the experiment, 0.5 ml vehicle (0.9 % NaCl) was given by femoral vein injection to sham-operated rats. After, their abdomens were opened by a midline incision, the celiac artery was isolated and waited for 90 min;

Group 2, the rats with ischemia-reperfusion (n=10), 5 min before the beginning of the experiment, 0.5 ml vehicle (0.9 % NaCl) was given intravenously to these rats. After 5 min, their abdomens were opened and the celiac artery was occluded with a small clamp for 30 min. Reoxygenation was allowed by removal of this clamp for 60 min;

Group 3, the rats with ischemia-reperfusion and L-carnitine treatment (100 mg/kg in 0.5 ml 0.9 % NaCl) (n=10), L-carnitine was injected and then performed ischemia-reperfusion as mentioned in group 2.

The animals were starved for 18 h prior to the experiments, but were allowed free access to water. At the end of fasting period, the rats were anesthetized by intraperitoneal injection of 1 g/kg urethane and their abdomens were opened by midline incision. Vehicle+I/R and L-carnitine+I/R groups were exposed to ischemia-reperfusion. The rats were sacrificed by exsanguination via the abdominal aorta under urethane anesthesia. The stomachs were removed rapidly and opened at the lesser curvature, extended in cold petri dishes after rinsing with ice-cold saline. To measure the degree of mucosal damage by ischemia-reperfusion in rats with L-carnitine pretreatment, the gastric mucosa were carefully examined macroscopically and microscopically.

Assessment of gastric mucosal injury index:

Quantitative histological assesment of mucosal injury was performed using the method described by Gundersen et al (22). The stomachs were opened through the lesser curvature and fixed on the petri dishes. Stomachs were examined under the microscope (Zeiss Stemi SV 11, x20) with Cavalieri Measurement Ruler. The sum of the areas of damage was calculated and results were expressed as total area of lesions (mm²).

Measurement of acidic mucopolysaccharide content in the gastric mucosa:

Acidic mucopolysaccharides, an indicator of the gastric mucosal barrier were measured, based on the alcian blue-binding capacity of the gastric mucosa. The alcian blue-binding capacity of gastric mucosa was measured using the method of Corne et al. (23). Stomach tissue was washed in ice-cold 0.25 M sucrose solution and was weighed to be incubated for 2 h in 10 ml 0.1 % alcian blue 8 6x dissolved in 0.16 M sucrose and buffered with 0.05 M sodium acetate, adjusted to pH 5.8

with HCl. All stomach tissues were transferred to 10 ml of 0.25 M sucrose, and two successive washes of 15 and 45 min, in 0.25 M sucrose were carried out. Thereafter, the dye complexes with mucus were eluted by immersion for 2 h in aliquots of 0.5 M $MgCl_2$, 10 ml/g of tissue, with occasional shaking and removed and the magnesium chloride solution was shaken briefly with 10 ml diethyl ether. The optical density of the aqueous layer was read at 605 nm. Results were expressed as micrograms per gram of tissue.

Measurement of PGE₂ content in gastric mucosa:

The assay of gastric PGE₂ levels were performed according to the method described by Cockrell and Ellis (24). PGE₂ content of the gastric mucosa was measured using a Varian HPLC (model 5000; Varian Instrument Group, Walnut Creek, CA, USA). Gastric mucosa scraped rapidly with a glass slide. Mucosal scrapings were weighed and homogenized at 3500 rpm for 60 sec in 2 ml chloroform. Mucosal extracts were dissolved in 1 ml mobile phase composed of water: acetonitril, benzene, acetic acid (767:230:2:1 v/v/v/v) and loaded onto a reverse phase column SP C 18 (150x4 mm, 3 μ m particle size). With a mobile phase flow rate of 1 ml/min, eluted mucosal extracts were detected by UV absorbance at 254 nm at 28°C. Quantitative integration of chromatographic separations were performed using a Varian (Model 4290) integrator (Varian Instrument Group, Walnut Creely CA, USA) and PGE₂ standard as a reference.

Measurement of gastric lipid peroxidation:

Thiobarbituric acid (TBA) reactans in the gastric mucosa, an index of lipid peroxidation, were measured by the method of Stocks et al (25). The mucosa of the stomach was scraped with a blunt knife and frozen on dry ice with tissue disruptor (TRI-R, STIR-R, model K43) driven at 9000 rpm 30 sn. A sample of 3 ml from each homogenate was added to 2 ml trichloroacetic acid (TCA) and centrifuged for 10 min at 4000 rpm. 3 ml of the clear supernatant was added to 1 ml thiobarbituric acid (TBA) and boiled for 15 min. The reagent 1,1,3,3 tetraethoxypropane was used as a standard. The reaction product was assayed spectrophotometrically at 532 nm. The results were expressed as nmol TBA per gram protein. Protein was determined by the method of Lowry (26).

Assay of gastric mucosal superoxide dismutase activity:

SOD activity in the gastric mucosa was assayed by the method described by Misra et al (27). Mucosal tissue was homogenized at 9000 rpm for 30 sec. Homogenates were centrifuged at 25000 rpm for 60 min at 4°C. Supernatants were added to reaction mixture (550 μ l HCO_3 buffer, 400 μ l EDTA, 500 μ l epinephrine) and SOD activity was measured at 480 nm. Adrenochrome was generated with epinephrine otooxidation. Since, the production of adrenochrome is inhibited by Cu/Zn SOD, Cu/Zn SOD is responsible from the change of absorbance at 480 nm at which the absorbance of adrenochrome is maximum. SOD activity was expressed as the amount of the SOD standard showing activity equivalent to the determined activity.

Assay of gastric mucosal catalase activity:

Catalase activity of gastric mucosa was assayed by the method of Aebi et al (28). The mucosal tissue was disrupted using a homogenizer (TRI-R, STIR-R, model 43) in 50 mM phosphate buffer (pH:7.0) and the homogenate was centrifuged at 3000 rpm for 15 min at 4°C. Decomposition of

H₂O₂ was determined by the decrease in absorbance at 240 nm. One unit of this activity was defined as the amount of enzyme decomposing 1 μmol/ H₂O₂ / per min.

Assay of gastric mucosal glutathione peroxidase (GPx) activity:

Gastric GPx activity was measured using a modification of the method of Paglia and Valentina (29). Gastric tissue was homogenized in the phosphate buffer (50mM, pH:7.2). Homogenates were centrifuged at 15000 rpm at 4°C (MR 1822 Heraus Biofuge Centrifuge). A sample of supernatant fluid was added to the reaction mixture (0.3 M EDTA, 0.1 mM NADPH, 0.5 U glutathione reductase, 0.5 mM NaN₃), t-butyl hidroperoxide (t-BOOH) and reduced glutathione (GSH). The absorbance at 340 nm was recorded for 3 min.

Reduced glutathione is oxidized by the GPx. Glutathione reductase is reduced to oxidize glutathione using to NADPH. One unit of GPx activity is defined as the amount of enzyme oxidizing 1 μ mol NADPH/min/g protein. The amount of protein was determined by the method of Lowry et al (20).

Statistics:

The results were expressed as mean ± SE. Statistical evaluations were performed by using the ANOVA test. Tukey test is used for post-hoc. P values greater than 0.05 were considered as insignificant.

RESULTS

Gastric mucosal injury:

As seen in *Fig.1*, the total area of the erosions was expressed a morphological index of gastric injury. In contrast to the normal color and appearance of the gastric mucosa of sham-operated rats, I/R caused erosions in gastric mucosa. After I/R, gastric mucosal lesion index was 27.28 ± 4.75 mm² ($p < 0.01$, vs sham-operated group). However, when rats were treated with L-carnitine, prior to I/R, the total area of mucosal erosions significantly reduced ($p < 0.05$ vs vehicle+I/R group).

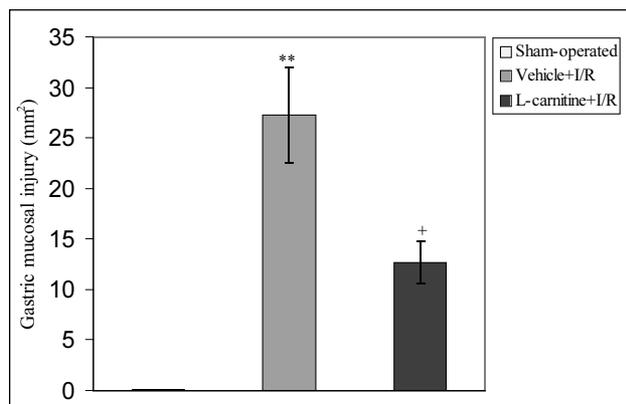


Fig 1. Effect of L-carnitine on acute gastric damage induced by ischemia-reperfusion (I/R). Carnitine (100 mg/kg body weight) was administered before I/R. The mucosal injury was expressed as the total area of mucosal lesions. Each bar represents means ± SE. ** $p < 0.01$ vs the value in sham-operated animals; + $p < 0.05$ vs the value in animals subjected to Vehicle+I/R.

Acidic mucopolysaccharide content of gastric mucosa:

The alcian blue-binding capacity, which indicates the acidic mucopolysaccharide content of gastric mucin, was $97.00 \pm 5.9 \mu\text{g/g}$ wet wt in sham-operated rats. Vehicle+I/R or L-carnitine+I/R groups did not change acidic mucopolysaccharide content of gastric mucosa. ($113 \pm 12.43 \mu\text{g/g}$ wet wt in vehicle+I/R group and $85.06 \pm 7.04 \mu\text{g/g}$ wet wt in I/R group exposed to L-carnitine) (Fig.2).

PGE₂ content of gastric mucosa:

PGE₂ content of gastric mucosa in sham-operated rats was $48.08 \pm 4.63 \text{ pM/g}$ wet wt. Ischemia-reperfusion caused a significant reduce in gastric mucosal PGE₂ ($24.00 \pm 1.66 \text{ pM/g}$ wet wt, $p < 0.001$ vs sham-operated group). The treatment with L-carnitine prevented the decrease in PGE₂ content of gastric mucosa due to I/R ($48.73 \pm 3.94 \text{ pM/g}$ wet wt, $p < 0.001$ vs vehicle+I/R group) (Fig.3).

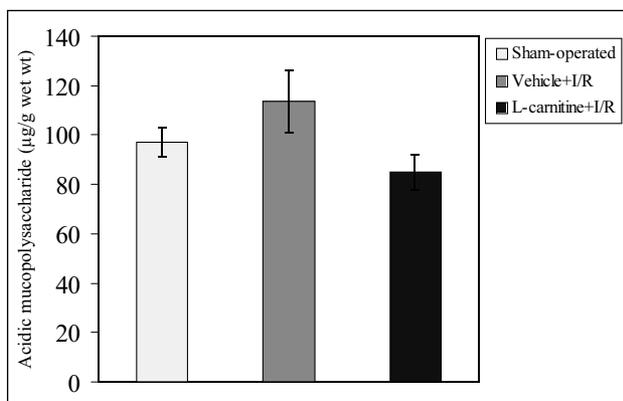


Fig 2. Effect of carnitine on acidic mucopolysaccharide content of gastric mucosa in rats subjected to ischemia-reperfusion (I/R). L-carnitine (100 mg/kg body weight) was administered before I/R. After I/R, acidic mucopolysaccharide was determined as described in 'Methods'. Each bar represents means \pm SE.

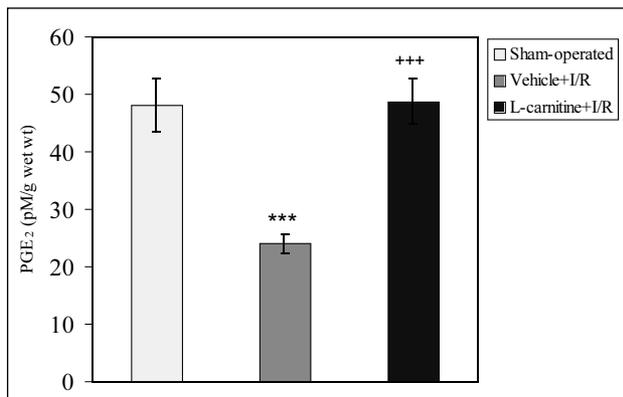


Fig 3. Effect of L-carnitine on gastric mucosal prostaglandin E₂ (PGE₂) in rats subjected to ischemia-reperfusion (I/R). L-carnitine (100 mg/kg body weight) was administered before I/R. After I/R, mucosal PGE₂ was determined as described in 'Methods'. Each bar represents means \pm SE. *** $p < 0.001$ vs the value in sham-operated rats; +++ $p < 0.001$ vs the value in animals subjected to Vehicle+I/R.

TBARS content of gastric tissue:

TBA-reactive substances in the gastric mucosa, the index of lipid peroxidation increased markedly after I/R injury from a basal concentration of 116.47 ± 9.17 nmol/g protein to 154.45 ± 10.79 nmol/g protein ($p < 0.05$). Treatment of rats with L-carnitine resulted with a significant reduction in TBARS content compared with rats exposed to I/R ($p < 0.05$, vs vehicle+I/R group) (Fig. 4).

Antioxidant capacity of gastric tissue:

As seen in table 1, vehicle+I/R group caused an obvious decrease in SOD and catalase activities in gastric tissue when compared with the findings of sham-operated rats ($p < 0.001$, $p < 0.001$, respectively). Following I/R, decreased catalase activity in gastric tissue was increased by pretreatment with L-carnitine ($p < 0.001$). SOD activity of tissue was unchanged in vehicle+I/R group with pretreatment L-carnitine when compared with the vehicle+I/R group. There was no significant difference in gastric mucosal GPx activity between all groups (Table.1).

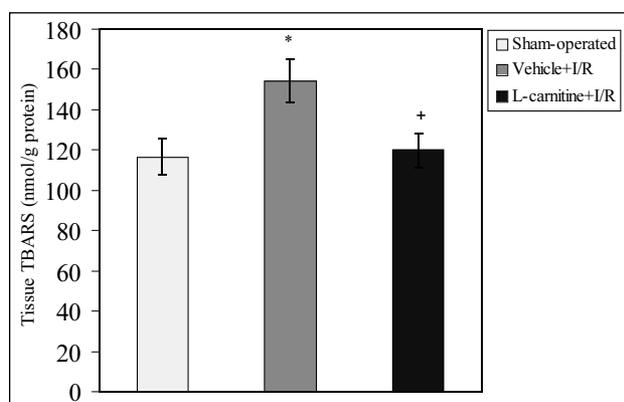


Fig 4. The effect of L-carnitine (100 mg/kg bw) on TBARS in I/R group. Each value represents the mean \pm SE. * $p < 0.05$ vs the sham-operated group, + $p < 0.05$ vs Vehicle+I/R group.

Table 1. Changes in gastric mucosal catalase, superoxide dismutase and glutathione peroxidase activities during ischemia-reperfusion without and with L-carnitine.

	Sham-operated (n=10)	Vehicle+I/R (n=10)	L-carnitine+I/R (n=10)
Catalase activity (k/g protein)	25.37 \pm 2.86	9.31 \pm 0.29***	28.07 \pm 1.21 ⁺⁺⁺
SOD activity (U/g protein)	16.44 \pm 2.18	8.12 \pm 0.23***	5.12 \pm 0.33
GPx activity (U/g protein)	13590.71 \pm 1104.6	14406.45 \pm 1932.7	18435.56 \pm 1728.72

*** $p < 0.001$, as compared with sham-operated rats. ⁺⁺⁺ $p < 0.001$, as compared with Vehicle+I/R rats. L-carnitine (100 mg/kg bw) were administered IV. Values are expressed as means \pm SE SOD (superoxide dismutase), GPx (glutathione peroxidase).

DISCUSSION

The results of this study showed that gastric injury was formed during 60 min reperfusion following 30 min of ischemia but pretreatment with L-carnitine protected the gastric mucosa of rats against the noxious effects of ischemia-reperfusion.

Previous studies indicated that I/R leads to marked injury in the rat gastric mucosa similar with our findings (1,10,13,30-32). Oxygen radicals have been reported to be involved in the pathogenesis of acute gastric lesions induced by I/R (14,30,33,34). We previously showed that L-carnitine had a protective effect on the harmful effects of stress induced by reactive oxygen species in the gastric mucosa (35). However, there are several reports about its antioxidant effects against oxidative stress in other tissues. It is demonstrated that antioxidant agents such as melatonin, allopurinol, vit C, vit E etc. decreased the total area of lesions induced by I/R in gastric mucosa (13-15, 36).

Ishii et al. have demonstrated that ROS and NO seem to be implicated in the ischemia-reperfusion injury and reduction of gastric ischemia-reperfusion injury may be via reduction of ROS and NO toxicity (37). However, gastric mucosal lesions in rats induced by I/R can be attenuated by SOD, catalase and NO-synthase inhibitors suggesting that ROS cooperate with NO in I/R-induced gastric damage and that reduction in ROS and NO toxicity is required to prevent the formation of acute gastric lesions by I/R. In this study, we did not demonstrate but the protective effect of L-carnitine against I/R induced gastric mucosal lesion may be due to inhibition of NOS activity. Koeck and Kremser demonstrated that L-carnitine causes a significant decrease of the activity of nitric oxide synthase (38). Calabrese et al. showed that L-carnitine causes a significant decrease of the activity of nitric oxide synthase (NOS) in patients with active multiple sclerosis (39).

It is known that oxygen radicals are the main source of hydroperoxides for the activation of PGH synthase. In this study, the production of PGE₂ was reduced by ischemia-reperfusion. The decrement of mucosal PGE₂ content was probably due to endothelial dysfunction induced by I/R in the gastric mucosa. Hence, we examined the effect of L-carnitine on PGE₂ production in the gastric mucosa of rats subjected I/R. L-carnitine prevented the decrease in PGE₂ content of gastric mucosa due to I/R. It is probable that the antioxidant activity of L-carnitine kindered the injurious effect of ROMs on endothelial integrity.

The gastric mucosal barrier is a complex system made up of submucosal epithelial and mucus elements (40). The mucus gel layer is a thick organized layer adherent to the epithelium and plays an important role in protection of the epithelium against acid, pepsin and mechanical damage (32,40,41). The stimulation of production of gastric mucin, PGE₂ and bicarbonate and the decrease of acid output help to maintain mucosal integrity (40). Prostaglandins exert potent protective effects and inhibition of prostaglandin formation abolishes gastroprotection, thus they have been proposed as key mediators in mucosal

defense. Although L-carnitine has beneficial effects on PGE₂ content of gastric mucosa no significant differences have been observed between gastric mucus of rats treated with L-carnitine before I/R. In contrary to our findings, Kitano et al. reported that the mucus layer was decreased during ischemia by microscopic observation (14). Kawai et al. showed that the reduction in mucosal mucus was induced by gastric ischemia and significantly recovered 60 min after reperfusion (41). In this study, the gastric mucus content was measured after 60 min following reperfusion; consequently, it was found constant.

Free oxygen radicals are accepted as mediators of gastric mucosal injury induced by ischemia-reperfusion (14,15,33,34). Free oxygen radicals initiate a free radical chain reaction known as lipid peroxidation and we showed that the gastric mucosal injury and TBARS increased significantly following ischemia for 30 min and reperfusion for 60 min. In this study, we demonstrated the preventive effect L-carnitine on gastric mucosal damage induced by lipid peroxidation. There are antioxidative enzymes such as GPx, SOD, CAT etc. which reduce increased lipid peroxidation levels in the gastric mucosa (2,7,13). It is well known that administration of the free radical scavengers or antioxidant agents prevented the mucosal injury in rats subjected to I/R. L-carnitine was found to be a scavenger for hydroxyl radicals and inhibited hydroxyl radical production in the Fenton reaction system (19). However, the preventive effect of L-carnitine on the formation of oxygen reactive species due to the xanthine/xanthine oxidase system has been shown by Di Giacomo et al. (18). Therefore, it can be hypothesized that the decrease in gastric mucosal blood flow induced by ischemia should cause hypoxia in gastric mucosa. In this way, the degradation of ATP into hypoxanthine and xanthine, which are substrates for xanthine oxidase, occurs in the gastric tissue (42). It is demonstrated that L-carnitine inhibits xanthine oxidase activity (18). In this manner, the administration of an inhibitor of xanthine oxidase enzyme may prevent rat gastric mucosal lesions induced by I/R.

We examined the effects of L-carnitine on the activities of antioxidant enzymes; SOD, GPx, CAT against tissue peroxidative damage. Our findings demonstrated that SOD and CAT activities significantly decreased in rats subjected to I/R and pretreatment with L-carnitine recovered this decrease in the CAT activity, but GPx activity maintained constant now during ischemia-reperfusion and the treatment with L-carnitine. Our results apparently agree with those obtained by Kwiecien et al. who showed a significant increase of MDA level after I/R, accompanied by decrease of enzymatic activity of antioxidative enzyme-superoxide dismutase (SOD) (30). Ferrari et al. have previously demonstrated that pretreatment with L-carnitine had no effect on the GPx activity in agree with our finding (17). Previously studies reported that mucosal SOD activity decreased during ischemia and the decrease in mucosal SOD activity was related to CAT activity (33,43-45). Bray et al. demonstrated that the decrease in the SOD activity was associated with the decrease in the CAT activity and elevated H₂O₂ levels due to the decrease of CAT activity inhibited SOD activity

(46). However, oxidative stress is known to cause the up regulation of antioxidant gene expression. Kaçmaz et al. hypothesized that the decreased antioxidant enzymes activities in ischemia-reperfusion may be mediated by down regulation of gene expression of antioxidant enzymes (43).

Our data suggested that L-carnitine prevents the occurrence of mucosal lesions by strengthening the gastric mucosal barrier. It acts by preventing the decrease in PGE₂ content induced by oxygen radicals in gastric mucosa. Furthermore, L-carnitine provides a marked protection against I/R induced gastric mucosal injury. In addition the increase at the activity of CAT seem to contribute significantly to the mucosal protection in stomach subjected to I/R. Taken together, these results support a role for endogenous L-carnitine in maintaining gastric mucosal integrity and have implications in the treatment of conditions involving reduced gastric mucosal barrier.

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