

EXPERIMENTAL STUDY

Role of curcumin in mesenteric ischemia – reperfusion injury in rats

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Abstract: *Background:* Curcumin is an antioxidant molecule that has been shown to attenuate ischemia/reperfusion (I/R) injury in several organ systems. In the present study, we aimed to evaluate the possible effects of curcumin on contractile response to agonists and histopathological alterations in rat esophagus subjected to mesenteric I/R.

Materials and methods: Adult male Wistar albino rats were randomly allocated to 4 groups, namely group I: sham-operated animals (n=10); group II: animals subdued to I/R injury only (n=10) and laparotomy; 45 minutes of superior mesenteric artery ligation were followed by 2 hours of reperfusion, group III: curcumin/sham (n=10); 20 days before I/R, curcumin (200 mg/kg/d) was administered by gastric gavage, and group IV: curcumin-I/R (n=10). Mesenteric ischemia/reperfusion model was generated by clamping the superior mesenteric artery for 45 min followed by reperfusion for 2 h. Oral administration of curcumin by gavage at a dose of 200 mg/kg/day lasted 20 days just before inducing the mesenteric ischemia. At the end of reperfusion period, all animals were sacrificed and esophagus samples were collected to assess the contractile response to agonists and histopathological alterations.

Results: Ischemia/reperfusion significantly decreased the contractile responses to carbachol and KCl and this decrease was attenuated by curcumin. Pretreatment with curcumin caused a remarkable decrease in histopathological parameters such as edema, congestion and inflammatory cells.

Conclusions: The results of the present study demonstrate for the first time that curcumin can attenuate the esophageal injury associated with I/R (Tab. 4, Fig. 3, Ref. 32). Full Text in PDF www.elis.sk.

Key words: contractile function, curcumin, esophagus, mesenteric ischemia.

Mesenteric ischemia is frequently encountered in multiple disease states and surgical procedures including cardiovascular interventions, small bowel transplantation mesenteric thrombosis, shock syndromes, and severe burns (1, 2). The ensuing reperfusion is unavoidable and may induce downstream tissue injury. Intestinal ischemia and reperfusion injury can accelerate interactions between endothelium and different cell types resulting in microvascular injury, cellular necrosis, and apoptosis (3–6). During mesenteric ischemia/reperfusion (I/R), reactive oxygen species (ROS) attack the cell membrane constituents and thus bring about lipid peroxidation, membrane disintegration, and increased microvascular permeability. The latter induces activation and adhesion of polymorphonuclear neutrophils (PMNs), release of proinflammatory substances, and increased formation of ROS. Activated neutrophils in this tissue enhance ROS and cytotoxic protein production, which leads to an induction of inflammatory cascade (3, 4) resulting in cell death and organ failure. The inten-

sity of this inflammatory reaction in postischemic tissue can be so great that the injury response to reperfusion is manifested also in distant organs. These remote effects of I/R are most frequently observed in the lung and cardiovascular system, and can possibly result in the development of systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS) (7). To counter these processes, various therapeutic strategies have been attempted to attenuate the mesenteric I/R injury including antileukocyte and anti-inflammatory therapies, as well as glutamine, glycine and antioxidant supplementation (5, 8).

Curcumin (diferuloyl methane) is a polyphenolic diketone found in *Curcuma longa* Linn. (Family Zingiberaceae). The rhizomes of the plant are a rich source of phenolic compounds, particularly curcumin. It possesses extensive pharmacological and therapeutic properties, including anticarcinogenic effects, and exhibits a potent antioxidant property (9–12). Curcumin is known to be a potent inhibitor of nuclear factor- κ B (NF- κ B). It has been shown to attenuate I/R injury in several organ systems. Recently, it has been reported that curcumin administration ameliorated I/R injury in rat kidney, myocardium, and nervous tissue (13). Curcumin treatment has been recently shown to attenuate reperfusion injury in a superior mesenteric artery I/R model in rats (14).

Despite numerous studies on the effects of I/R on the mesenteric smooth muscle responses, its probable effect on esophageal smooth muscle responses has not been delineated. Therefore, the

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present study was designed to investigate the effects of I/R on rat esophageal responses and the role of curcumin supplementation.

Materials and methods

Chemicals

Carbachol was purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA) and dissolved in double distilled water. Compounds used for preparing the Tyrode's solution, were purchased from Merck (Merck KGaA, Darmstadt, Germany).

Animals

In this study, 40 male Wistar-Albino rats weighing between 280 and 300 gr were used. Animals were kept under standardized conditions; 20 ± 2 °C, 50 ± 5 % humidity, 12/12 light-dark cycles at Selçuk University Experimental Medicine Research and Application Center. The rats were fed a standard laboratory diet with water *ad libitum*. The local ethics committee of our institution approved this study. The animals were randomly divided into four groups; ten animals in each groups. Sham: animals exposed to laparotomy without clamping the superior mesenteric artery (SMA); I/R (ischemia-reperfusion): animals exposed to laparotomy with occlusion of SMA for 45 min, followed by 120 min of reperfusion period; Curcumin/Sham: 200 mg/kg of curcumin as described before (15–17) was given with oral gavage for 20 days to animals who were thereafter exposed to laparotomy without clamping the SMA; Curcumin/I/R: 200 mg/kg of curcumin was given with oral gavage for 20 days to animals who were thereafter exposed to laparotomy with occlusion of SMA for 45 min, followed by 120 min of reperfusion period.

Ischemia-reperfusion protocol

Animals were anesthetized by intraperitoneal injection of ketamin hydrochloride plus xylazin. The abdomen was opened with a midline incision. The intestinal I/R injury was established by occluding the SMA with atraumatic microvascular clip for 45 min followed by 120 min reperfusion. Ischemia was recognized by absence of pulse or pale color of intestine. Following ischemia, the microvascular clamp was removed and reperfusion was confirmed by restoration of pulsation and color prior to closing the incision. Sham-operated animals were submitted to the abdominal incision without I/R.

Curcumin supplementation

Curcumin (*Curcuma longa*; Turmeric) was purchased from Sigma Aldrich (C1386). Curcumin dissolved in corn oil (Sigma C8257). Rats in Curcumin/Sham and Curcumin/I/R groups were given 200 mg/kg of curcumin with oral gavage for 20 days.

Preparation of esophagus

The rats were harvested by decapitation and almost 1.5 cm of thoracic portion of esophagus was excised. Esophageal smooth muscle strips were prepared and mounted in 20 ml organ chambers for isometric tension measurement. The organ chambers contained Tyrode's solution composed of (mmol l^{-1}): NaCl 136.0; KCl 2.7; CaCl_2 1.8; MgCl_2 1.05; $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ 0.42; NaHCO_3 11.9; glucose

5.5. The solution was gassed with 95 % O_2 and 5 % CO_2 during the study, and the temperature was maintained at 37 °C. The strips were connected to a force transducer (BIOPAC MP36, Santa Barbara, California, USA) connected through amplifiers to a ITBS08 Integrated Tissue Bath System (Commat, Ankara, Turkey). The strips were allowed to equilibrate for 90 min under a resting tension of 0.5 g before the experiments began. During this period the bath fluid was routinely changed every 15 min.

Esophageal contractility

At the end of the equilibration period, carbachol (10^{-9} – 3×10^{-4} M) was added in a cumulative manner to the organ baths.

In another part of the study the same procedure was applied with cumulative addition (5–100 mM) of KCl to the organ baths. Each experiment was performed with a tissue sample taken from one animal.

Histopathologic examination

Immediately after death, the tissues in a 10% formaldehyde solution were processed in a cassette autotechnic tissue-processing equipment (Leica ASP 300). When the processing was completed, the tissues were embedded in paraffin blocks and sections (5 μm in thickness) taken by microtome instrument onto lysine laminin. The preparations stained with hematoxylin and eosin were evaluated by Olympus BX51 microscope.

The histopathological evaluation of tissue damage included the intensity of inflammatory cell infiltration, edema, congestion, hemorrhage erosion, muscular degeneration, necrosis and fibrosis. Each parameter was scored between 0 and 3 (0: normal, 1: mild, 2: moderate and 3: severe).

Biochemical Analysis

Interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α) levels were analyzed in serum samples by using commercially available kits (BenderMed System, Vienna, Austria) according to manufacturer's instructions. Myeloperoxidase (MPO) activity was analyzed in intestinal tissue to determine ischemia by using a commercially available kit (Hycult Biotechnology, Uden, The Netherlands) according to manufacturer's instructions. The protein content of tissues was determined by the method of Lowry et al (18).

Statistical analysis

Concentrations of contractile agents causing 50 % of the maximal response (EC_{50}) were calculated from each individual concentration-response curve. The maximum effect values were calculated as a percentage of maximum response of tissue to carbachol and potassium chloride. Maximal responses and EC_{50} values for curves obtained from each groups were compared by using the unpaired or paired Student's t test, as appropriate. The difference was assumed to be significant at $p < 0.05$.

The histopathological results were expressed as median (min-max). All numerical data were first analyzed using the nonparametric Kruskal-Wallis test (whether there was a difference between groups) and then the Mann-Whitney U-test was performed to analyze two groups consecutively.

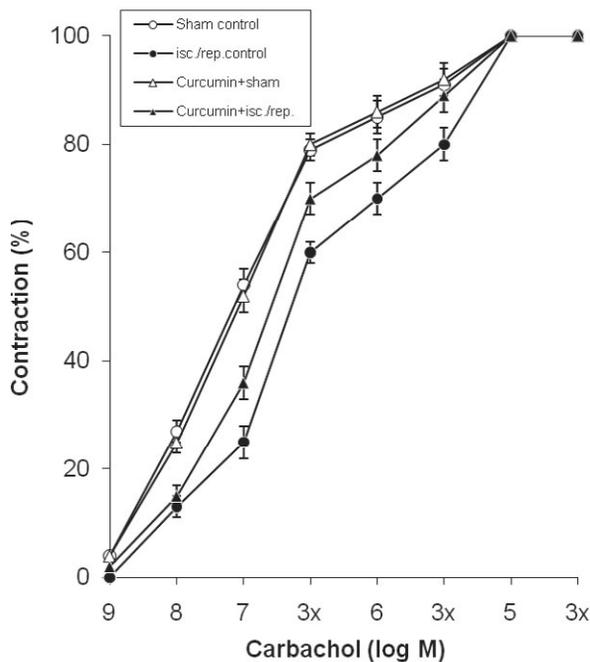


Fig. 1. Carbachol concentration-response curves of esophagus from sham control rats, I/R control rats, curcumin-treated rats and curcumin-treated I/R rats. Each point is the mean \pm SEM of six experiments.

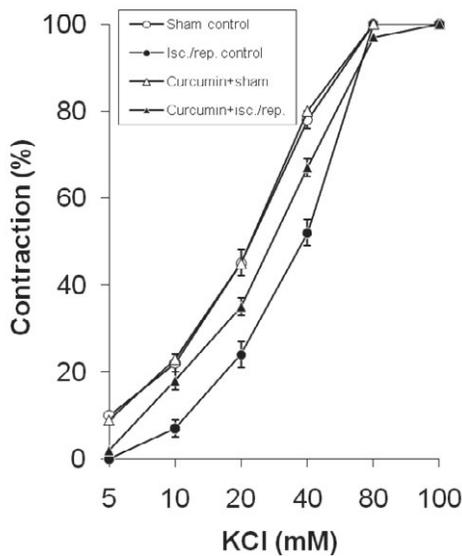


Fig. 2. KCl concentration-response curves of esophagus from sham control rats, I/R control rats, curcumin-treated rats and curcumin-treated I/R rats. Each point is the mean \pm SEM of six experiments.

Results

Esophagus contractility

The addition of carbachol at concentration from 10^{-9} M to 3×10^{-4} M into organ bath fluid resulted in a dose-dependent contractile effect on the esophageal strips from all groups, providing

Tab. 1. EC₅₀ values for carbachol and KCl of esophagus from rats. Each value is derived from six experiments. Data are means \pm SEM.

	Carbachol ($\times 10^{-7}$ M)	KCl ($\times 10^{-2}$ M)
Sham control	7.0 \pm 0.1	2.3 \pm 0.1
Isc./rep.	2.2 \pm 0.1 ^a	3.8 \pm 0.1 ^a
Curcumin	8.6 \pm 0.2 ^b	2.2 \pm 0.2 ^b
Curcumin+isc./rep.	1.6 \pm 0.2 ^b	2.8 \pm 0.1 ^b

^a p<0.05 compared to Sham control, ^b p<0.05 compared to Isc./rep.

Tab. 2. E_{max} (g) values for carbachol and KCl of esophagus from rats. Each value is derived from six experiments. Data are means \pm SEM.

	Carbachol	KCl
Sham control	4.5 \pm 0.1	3.0 \pm 0.1
Isc./rep.	2.9 \pm 0.1a	1.6 \pm 0.1a
Curcumin	4.9 \pm 0.2b	3.3 \pm 0.2b
Curcumin+isc./rep.	5.3 \pm 0.2b	4.3 \pm 0.1b

^a p<0.05 compared to Sham control, ^b p<0.05 compared to Isc./rep.

sigmoid curves (Fig. 1). The dose-response curve obtained from tissues of animals subjected to I/R without administrating anything shifted to the right. In other words, the contractile responses induced by carbachol were significantly and dose-dependently inhibited by the induction of IR (p<0.05). E_{max} value for carbachol in the I/R control group was significantly lower than in the sham-operated control group (Tab. 1). The IR-induced reduction in contractility was significantly ameliorated by pretreatment with curcumin. Treatment of sham animals with curcumin has provided the same contractile response curve with similar E_{max} value as sham alone group does.

In response to cumulative concentrations of KCl ranging from 2 mM to 10 mM, esophagus strips contracted in a dose-dependent fashion in all groups, rendering sigmoid curves with EC₅₀ and E_{max} values (Fig. 2). The contractile response induced by KCl was significantly and dose-dependently inhibited by the induction of I/R. Reduced contractility due to I/R was significantly ameliorated by curcumin pretreatment. Pretreating sham animals caused similar dose-response curve to sham-operated group in respect of EC₅₀ and E_{max} values.

Histopathological findings

Based on histopathological analysis of 6 sections for each group, the most extensive changes in morphology were evident in I/R control group (Fig. 3). Edema, congestion, and inflammatory cell infiltration were clearly observed and noticed as grades 2 and 3. In sham-operated control group, no pathological change was detected as demonstrated in picture A; grading as 0. The analysis also showed that the pretreatment with curcumin restored remarkably the typical alterations in morphology as depicted in Table 2. Sections obtained from curcumin-pretreated sham group were observed with normal morphological appearance, identical to those from sham-operated control group (Tab. 3).

Biochemical findings

Serum IL-6 and TNF- α levels were not different among the groups. Intestinal MPO activity was lower in Curcumin/Sham group compared to that in IR group (p<0.05) (Tab. 4).

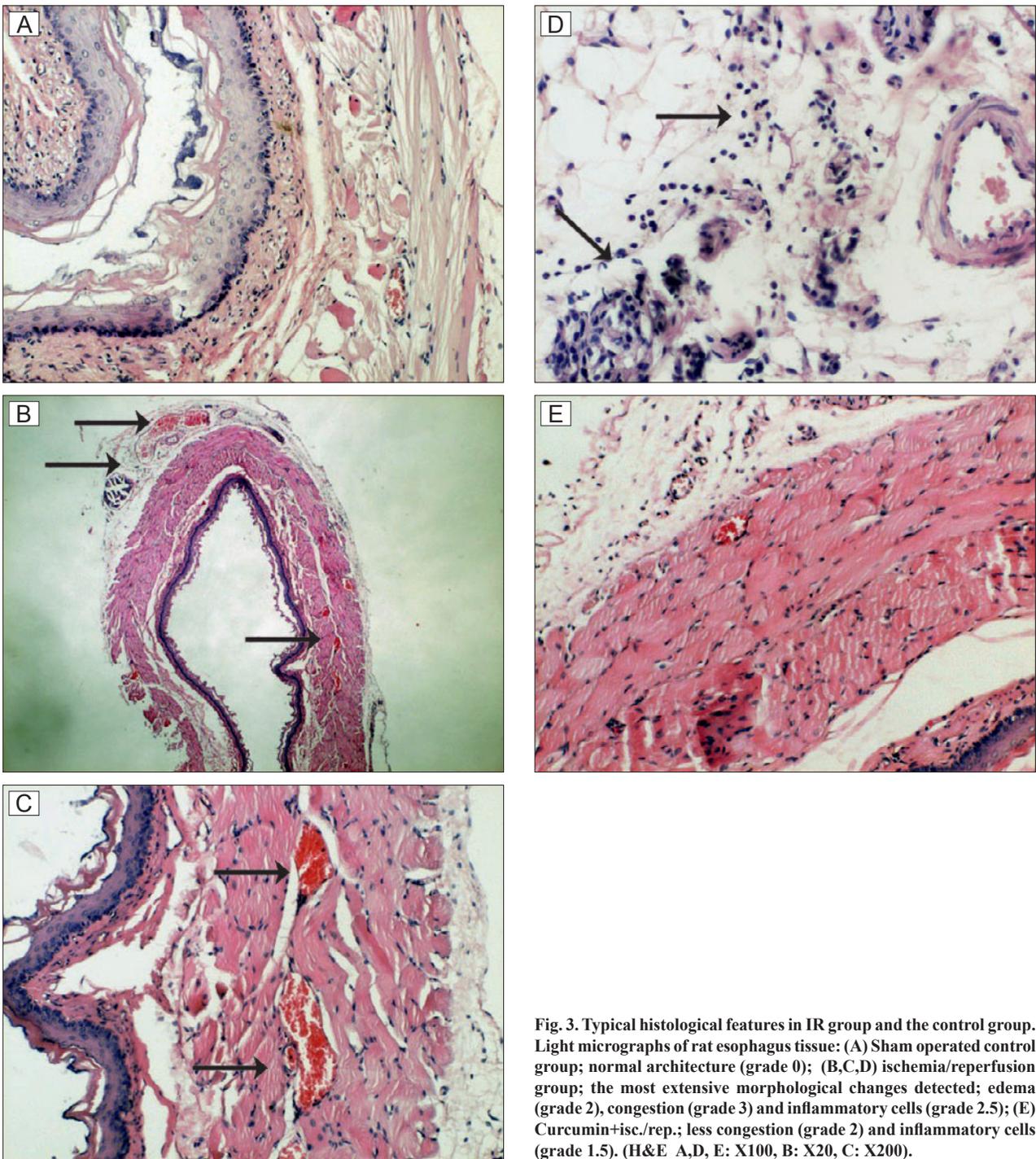


Fig. 3. Typical histological features in IR group and the control group. Light micrographs of rat esophagus tissue: (A) Sham operated control group; normal architecture (grade 0); (B,C,D) ischemia/reperfusion group; the most extensive morphological changes detected; edema (grade 2), congestion (grade 3) and inflammatory cells (grade 2.5); (E) Curcumin+isc./rep.; less congestion (grade 2) and inflammatory cells (grade 1.5). (H&E A,D, E: X100, B: X20, C: X200).

Discussion

The present study demonstrated that mesenteric I/R resulted in a decreased esophageal contractility in response to both carbachol, receptor-mediated induction, and KCl, non-receptor-mediated induction. Decreased contractile response observed also in non-receptor-mediated induction supports the possibility that

ischemia/reperfusion may not alter the ligand-receptor interaction but rather changes the regulation of postreceptor processes (i.e. excitation-contraction coupling) (19). Our data are consistent with these findings. This study also reveals that curcumin pretreatment prevents the esophageal dysfunction and demonstrates a protective effect against esophageal damage resulting from mesenteric ischemia-reperfusion. This is the first report to show that curcumin

Tab. 3. Comparison of histological damage of rat esophagus median (min-max).

	Edema	Congestion	Inflammatory cells	Haemorrhage	Erosion	Necrosis	Fibrosis	Muscular degeneration
Control	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)
I/R	2* (2–2)	3* (2–3)	2.5* (2–3)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)
Curcumin/Sham	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)
Curcumin/I/R	1** (1–1)	2** (1–2)	1.5** (1–2)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)

*p<0.05 compared with control group. **p<0.05 compared with I/R group.

Tab. 4. Effects of curcumin supplementation and intestinal IR on serum IL-6 and TNF- α levels and intestinal MPO activity (mean \pm SD).

	Control	IR	Curcumin/Sham	Curcumin/IR
IL-6 (pg/mL)	349.00 \pm 165.92	456.00 \pm 164.98	369.21 \pm 189.99	322.94 \pm 175.76
TNF- α (pg/mL)	154.26 \pm 6.23	147.94 \pm 13.62	144.05 \pm 10.82	156.75 \pm 20.28
MPO (ng/mg protein)	304.53 \pm 89.42	363.20 \pm 67.74	253.98 \pm 52.46*	316.97 \pm 106.37

*p<0.05 compared to the IR

prevented an esophagus dysfunction induced by I/R. In this study, esophageal injury was assessed by histological method.

Oxidative stress plays an important role in mesenteric I/R injury. I/R injury leads to release of ROS or reactive nitrogen species (RNS) such as superoxide anion, hydroxyl radical, hydrogen peroxide, and peroxynitrite (20). During reperfusion, when the oxygen is re-supplied, tissue injury could be further exacerbated depending on the duration and intensity of ischemia. Meanwhile, it produces an excess of xanthine oxidase and oxygen free radicals, which would aggressively and indiscriminately damage the cellular macromolecules including DNA, proteins, and lipids (21). As it has previously been demonstrated, the development of I/R injury is associated with different representative pathologic processes such as congestion, hemorrhage, neutrophil accumulation and lipid peroxidation (22). In this study, edema, congestion and inflammation were clearly observed in mesenteric I/R group.

Aiming to improve the survival after acute mesenteric ischemia, a number of experimental studies have been carried out in order to test several pharmacological agents that might attenuate reperfusion injury (23). Curcumin is an active ingredient of turmeric, and is a polyphenolic compound that is known for its potent antioxidant capacity (24). Several factors support the sense of studies on the effect of curcumin administration on ischemia-related disorders, namely including the fact that curcumin is a naturally occurring substance. It is non-toxic to humans, tolerated as oral doses of up to 8000 mg/day, and evidenced safe by FDA approval (25). It was also noticed that curcumin is relatively inexpensive.

A protective effect of curcumin against hepatic I/R injury has been reported by a mechanism related to its ability for over expression of antioxidant enzymes (26). These factors together with the promising results of curcumin protective efficacy in hepatic and cardiac I/R injury prompted us to evaluate and discover the immune-mediated mechanism of its efficacy in protection against renal I/R injury and subsequent distant organ injury. However, the protective mechanism of curcumin effect on organ ischemia/reperfusion is not fully understood. Pre-treatment with curcumin was found to markedly attenuate the I/R-induced acute lung injury, probably through improving the oxidative stress (27). Furthermore, it has been shown that curcumin exerts a protective effect in several models of oxidant damage (28–30) including isoproterenol-

induced cardiac injury (31) and I/R injury in rabbits (13). Moreover, seven days of oral curcumin supplementation (3.6 g/d) has been shown to decrease the number of oxidative DNA adducts in malignant colorectal tissue suggesting that curcumin taken orally may reach sufficient concentrations in the gastrointestinal tract (32). In our study, curcumin pretreatment reduced the histopathologic indices such as edema, congestion and inflammatory cells caused by mesenteric I/R. Modulation of inflammatory response following I/R injury is an important component of tissue defense, mostly because inflammation is the major component of cell death. Similarly, Karatepe et al (14) reported that curcumin pretreatment reduced the histopathologic indices of intestinal mucosal injury caused by mesenteric I/R. The investigators also reported that addition of curcumin to nutritional supplements reduced I/R injury through a mechanism suppressing the oxidative stress.

In the present study, not only did we observe a decrease in edema, congestion and inflammatory cells, we also recorded a concomitant improvement in contractile response in case of curcumin pretreatment. The findings of the present study revealed that systemic administration of curcumin improved remarkably the non-receptor-mediated (e.g. KCl-induced) and receptor-mediated (e.g. carbachol-induced) significant reduction in esophageal contractions caused by I/R while the latter improvement even approached the sham-control levels.

Consequently, the present study is the first to show that curcumin treatment restores the reduction in esophageal smooth-muscle responses to carbachol and KCl in mesenteric I/R model. Our results suggest that curcumin treatment may ameliorate the structural and functional damage observed in experimental I/R, mainly due to reducing the edema, congestion and inflammation. Nevertheless, further studies are required to understand better the exact mechanism responsible for the protective effect of curcumin.

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