RESEARCH ARTICLE

β-carotene protects the gastric mucosa against ischemia-reperfusion injury in rats

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ABSTRACT. ***Background/aim:*** **The aim of the present study was to investigate the protective effect of β-carotene on gastric mucosal lesions caused by ischemia-reperfusion (I/R) injury in rat. Forty male rats were randomly divided into sham, control (I/R injury) and three β-carotene-pretreated groups. To induce the I/R lesions, the celiac artery was clamped for 30 min. The clamp was then removed to allow reperfusion for three hours. Pretreated-rats received β-carotene (15, 30 or 60 mg/kg daily, i.p.) or vehicle for five days before the induction of the I/R injury. Samples of gastric mucosa were collected to measure the mRNA expression of IL-1β, TNF-α and TGF-β by quantitative, real-time PCR. Pretreatment with β-carotene decreased the total area of gastric ulcer and mRNA expression, as well as plasma levels of pro-inflammatory cytokines, IL-1β and TNF-α, in a dose-dependent manner. The gene expression and plasma levels of the anti-inflammatory cytokine, TGF-β, were significantly increased in β-carotene-pretreated groups compared with the control. Our findings showed that the protective effect of β-carotene may be mediated partly by reducing mRNA expression and plasma levels of IL-1β and TNF-α, and concurrently, by increasing gene expression and plasma levels of the anti-inflammatory cytokine TGF-β. These findings suggest that β-carotene has a protective role in gastric mucosa. Further clinical and *in vivo* studies need to be undertaken to support this hypothesis.**

Key words: **β**-carotene, IL-1**β**, ischemia-reperfusion injury, quantitative real-time PCR, TGF-**β**

It is well established that β-carotene has antioxidant and anti-inflammatory properties. Several studies have demonstrated that serum levels of α- and β-carotene are inversely related to inflammatory markers such as C-reactive protein (CRP), soluble intercellular adhesion molecule-1 (sICAM-1) and IL-6 [1, 2]. Most carotenoids possess bioactivity related to mediation of anti-inflammatory responses, which can lead to a reduction of the risk of cardiovascular disease [3]. Dietary carotenoids have been shown to decrease the risk of certain types of immune system diseases, such as asthma and atopic dermatitis [4, 5].

Abréviations

I/R injury	ischemia-reperfusion injury
IL-1β	interleukin-1β
TNF-α	tumor necrosis factor-α
TGF-β	transforming growth factor-β
IL-10	interleukin-10
i.p	intraperitoneal
CRP	C-reactive protein
sICAM-1	soluble intercellular adhesion molecule-1
GAPDH	glyceraldehyde-3-phosphate dehydrogenase

Beneficial effects of pretreatment with β-carotene have also been shown in different I/R injury models such as renal [6], and hepatic systems [7]. To our knowledge, no previous study has specifically investigated the possible protective effect of β-carotene on gastric mucosa following I/R injury in rat. Therefore, the aims of the present study were to evaluate the effect of β-carotene on gastric mucosal lesions induced by I/R injury in rats, and to determine the possible role pro-inflammatory cytokines [IL-1β and TNF-α] and the anti-inflammatory cytokine, TGF-β, as well as their plasma levels after pretreatment with varying concentrations of β-carotene in a rat gastric mucosa I/R injury model.

METHODS AND MATERIALS

Animals

Male Wistar rats (body weight 160-220 g) were purchased from the animal house of the Ahvaz Jundishapur University of Medical Sciences. The animals were fed on conventional

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diets, and had free access to tap water. They were maintained under standard conditions of humidity and temperature (22 ± 2 °C), and a 12 h:12 h light/dark cycle. The animals were deprived of food, but not water, for 24 h before the experiment. All experiments were carried out in accordance with ethics committee of the Ahvaz Jundishapur University of Medical Sciences.

Animal grouping and surgical procedures

Forty rats were randomly assigned to one of five groups (n = eight per group): sham, positive control (gastric ischemia-reperfusion; I/R injury) and three, β-carotene-pretreated groups. Gastric I/R injury was induced according to the method of Wada [8]. Briefly, having been anesthetized with a mixture of ketamine and xylasine (60+15 mg/kg, i.p.), the rats underwent a midline laparotomy, and the celiac artery was carefully isolated from its adjacent tissues. The celiac artery was clamped using a ligature for 30 min to induce ischemia, and the ligature was then removed to allow reperfusion for three hours. Sham-operated rats underwent laparotomy without induction of I/R injury. Control rats received vehicle (Tween-80 in physiological saline, 4 mL/kg, i.p.) for five days before I/R injury was induced. To investigate the gastroprotective effect of β-carotene against mucosal damage induced by I/R injury, three groups of animals received β-carotene (i.p.) at doses of 15, 30 or 60 mg/kg daily for five days prior to the experiment; the last dose of β-carotene was given 24 h before the I/R injury induction [6]. At the end of the experimental period, the animals were killed by cardiac exsanguination. Blood samples were collected in chilled tubes containing EDTA, and were centrifuged at 3,000 rpm for five min. Separated plasma samples were kept at -80 °C until measurement of cytokine plasma levels. In order to assess the gastric mucosal lesions, the stomachs of the animals were removed, opened along the greater curvature, rinsed with physiological saline and pinned out in ice-cold saline. To calculate the size of the gastric lesions, the length (mm) and width (mm) of the mucosal ulcers were measured. The ulcer index (UI) was calculated using following formula:

$$UI\ (mm^2) = \text{length (mm)} \times \text{width (mm)} \times \pi/4 \text{ [9]}$$

Immediately after measurement of the surface area of the gastric lesions, 50 mg of gastric mucosal tissue, including the ulcer area and the surrounding ulcer margin, were quickly excised, snap-frozen and stored in liquid nitrogen for mRNA analysis.

Assay of cytokines

To investigate the effect of β-carotene on plasma levels of cytokines, an enzyme-linked immunosorbent assay (ELISA) method was employed using rat ELISA kits for measurement of IL-1β, TNF-α and TGF-β (eBioscience, Vienna, Austria), according to the manufacturer's instructions.

RNA extraction and cDNA synthesis

Total RNA was extracted from the frozen tissue samples using TriPure reagent isolation (Roche, Diagnostics). The concentration and purity of the total RNA was determined spectrophotometrically at wavelengths of 260 and 280 nm (Eppendorf, BioPhotometer *Plus*, Germany). The cDNA was synthesized from one microgram of the total RNA using the QuanTitect reverse transcription kit (Qiagen), according to the manufacturerĭs instructions.

Quantitative real-time PCR

Cytokine mRNA levels and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were measured using quantitative, real-time PCR (qPCR) using step-one systems (Applied Biosystems, USA). The specific primers (Bioneer, South Korea) for measurement of IL-1β, TNF-α, TGF-β and GAPDH were used [10] and are listed in *table 1*. All PCR amplifications were

Table 1
Specific primers for GAPDH and cytokine mRNA expression analysis.

Primer sequence	Amplicon size (bp)	Annealing/extension (°C)	Amplicon *T*m (°C)	Accession No.
TGF-β_1				
F: 5′-GCT-AAT-GGT-GGA-CCG-CAA-CAA-C-3′	100	64	79.54 ± 0.05	X52498
R: 5′-CAC-TGC-TTC-CCG-AAT-GTC-TGA-C-3′				
IL-1β				
F: 5′-AAT-GAC-CTG-TTC-TTT-GAG-GCT-GAC-3′	115	62	83.09 ± 0.09	M98820
R: 5′-CGA-GAT-GCT-GCT-GTG-AGA-TTT-GAA-G-3′				
TNF-α				
F: 5′-TGT-GCC-TCA-GCC-TCT-TCT-CAT-TC-3′	108	64	85.41 ± 0.16	X66539
R: 5′-CAT-TTG-GGA-ACT-TCT-CCT-CCT-TG-3′				
GAPDH				
F: 5′-TGC-TGG-TGC-TGA-GTA-TGT-CGT-G-3′	101	60	85.03 ± 0.08	M17701
R: 5′-CGG-AGA-TGA-TGA-CCC-TTT-TGG-3′				

* The standard errors of amplicons' Tm are smaller than 0.16 °C.

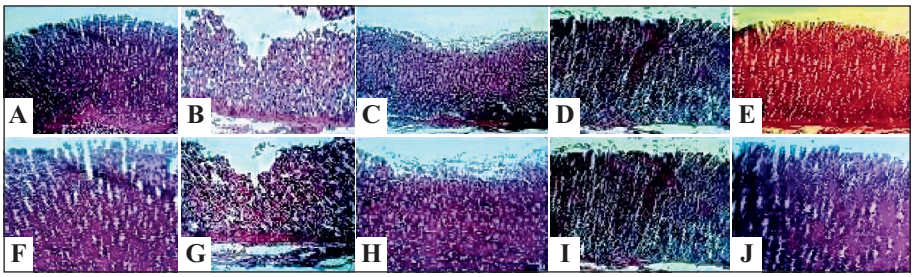

Figure 1

Histological evaluation of gastric mucosa. Representative gastric sections were obtained 3.5 h after sham-operated surgery or ischemia/reperfusion (I/R). A &F: Sham-operated group shows normal gastric mucosal tissue; B & G: Control (I/R) group indicate severe disruption to the upper half of mucosal thickness, and necrotic lesions penetrating deeply into mucosa; C & H: Rats pretreated with β-carotene (15 mg/kg, for five days before intervention), demonstrate moderate disruption of the surface epithelium; D & I and E & J: Rats pretreated with β-carotene (30 and 60 mg/kg, for five days before intervention), depict no disruption to the surface epithelium. All of the sections stained with hematoxylin and eosin; (A-E) ×100 magnification and (F-J) ×200 magnification.

performed in duplicate reactions and in a final volume of 20 μL containing 2 μL cDNA, 50 nm of specific primers and 10 μL of master mix SYBR green (2× qPCR Master mix with SYBR green I and Rox; Primer Design, England) using the following protocol: incubation at 95 °C for 10 min to activate DNA Taq polymerase, and 40 two-step cycles for 15 s at 95 °C for denaturation, 60 s at an annealing/extension temperature that is shown in *table 1*. In addition, the no-template negative control (H_2O) was routinely run in every PCR. The melting curve was examined at the end of the amplification process to ensure the specificity of the PCR products. The purity of each amplicon for each reaction was further confirmed by agarose gel electrophoresis. Expression levels of all cytokine genes were normalized against GAPDH expression (internal calibrator for equal RNA template loading and normalization). To determine the relative quantification of gene expression, the comparative cycle of threshold (Ct) method with arithmetic formulae ($2^{-\Delta\Delta Ct}$) was used [11].

Histological evaluation

For histological evaluation, stomachs from sham, control and β-carotene-treated animals were fixed in 10% formalin, dehydrated in grade ethanol, and embedded in paraffin. Thereafter, sections of tissue were cut at 5 μm using a microtome, stained with hematoxylin and eosin, and assessed under an Olympus microscope (IX50).

Statistical analysis

Data are shown as mean ± S.E.M. Statistical analysis was performed using one-way ANOVA, followed by *post hoc* Tukey's test. Significance was set at a $P<0.05$ level.

RESULTS

Effect of pretreatment with β-carotene on gastric mucosal lesions induced by I/R injury

Histological examination showed gastric lesions such as multiple erosions, exfoliation and necrosis of superficial cells, hemorrhages in the mucosal layer, and severe alterations in the architecture of glandular parts of the gastric mucosa after 3 h of reperfusion following 30 min of ischemia in control rats as compared with sham-operated animals (*figures 1B,G*). No damage was observed in the gastric mucosa of the normal rats in the sham-operated group (*figures 1A,F*). Pretreatment with β-carotene attenuated the gastric lesions induced by I/R injury (*figures 1C,E*). As shown in *figure 2*, the total area of the lesions induced by I/R injury was significantly decreased by pretreatment with β-carotene in a dose-dependent manner. The results also showed that β-carotene at 30 mg/kg was the optimal protective dose (*figure 2*).

Effect of pretreatment with β-carotene on mucosal mRNA expressions of IL-1β, TNF-α and TGF-β

The levels of mRNA expression of IL-1β and TNF-α in control rats were higher than in β-carotene-pretreated and sham-operated animals. These levels were significantly decreased in β-carotene-pretreated rats in a dose-dependent fashion compared with the control group

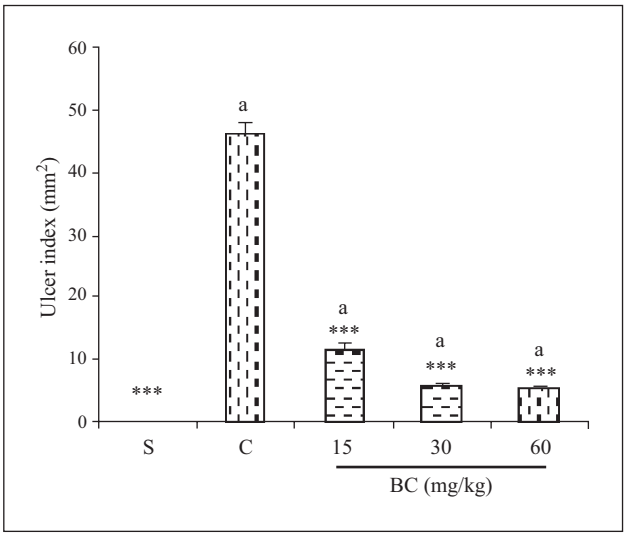

Figure 2

A graghic represenation of the ulcer index following ischemia-reperfusion injury among various treatment groups: S:sham, C: control and BC: β-carotene treated (15, 30 and 60 mg/kg, for five days prior to intervention). β-carotene produced a sigificant dose-dependent reduction in ulcer index. ***$P<0.001$ *versus* the control group and $^{a}P<0.01$ *versus* the sham group. Data are expressed as mean±S.E.M.

(*figures 2A,B*). The gene expression of TGF-β was significantly increased by pretreatment with β-carotene (*figure 2C*). The representative bands for cytokines studied; IL-1β, TNF-α, TGF-β and housekeeping mRNA are also shown in *figure 3*.

Effect of pretreatment with β-carotene on plasma levels of IL-1β, TNF-α and TGF-β

IL-1β and TNF-α plasma levels in control rats were higher than in β-carotene-pretreated and sham-operated

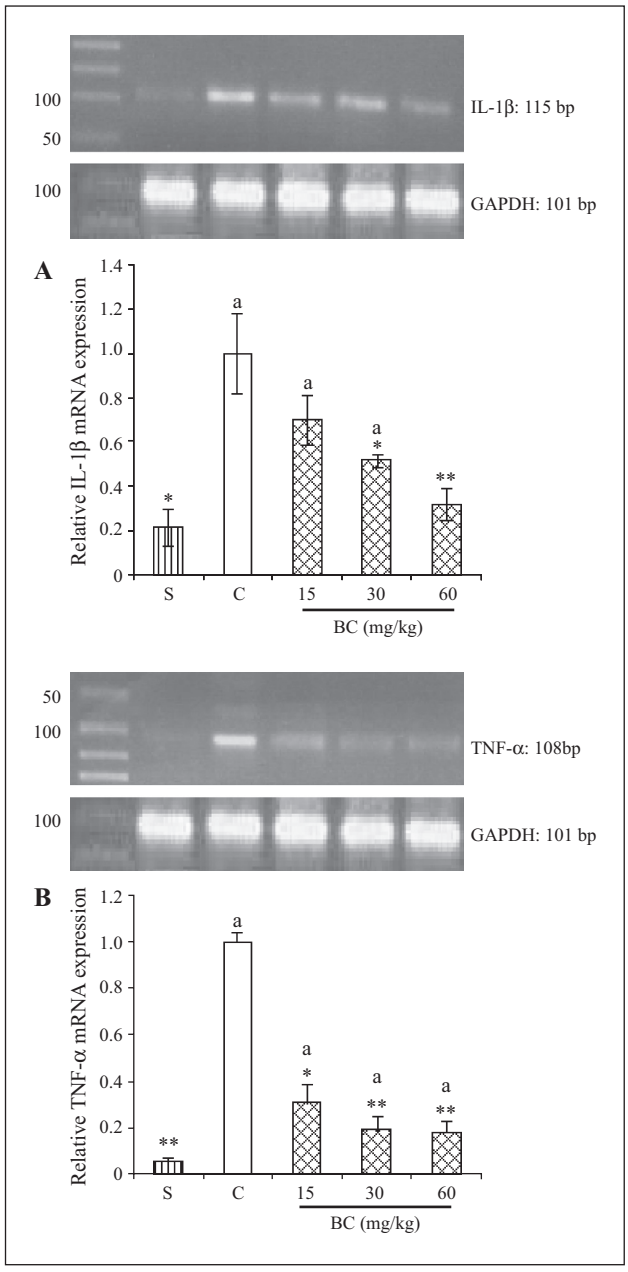

Figure 3
Graph and representative agarose gel bands (for target and housekeeping genes) of the effect of pretreatment with β-carotene (15, 30 and 60 mg/kg, for five days) on the gastric mucosal mRNA expression of (A) IL-1β, (B) TNF-α and (C) TGF-β. Analysis of qPCR results showed that the pre-administration of β-carotene decreases the mRNA expression of IL-1β and TNF-α. The mRNA expression of TGF-β was increased by β-carotene pretreatment (C). S:sham, C:control, BC: β-carotene; *P<0.05 and **P<0.01 *versus* the control group and [a]P<0.01 *versus* the sham group; significant increase (*P<0.05, **P<0.01) as compared to control. Data are expressed as mean±S.E.M.

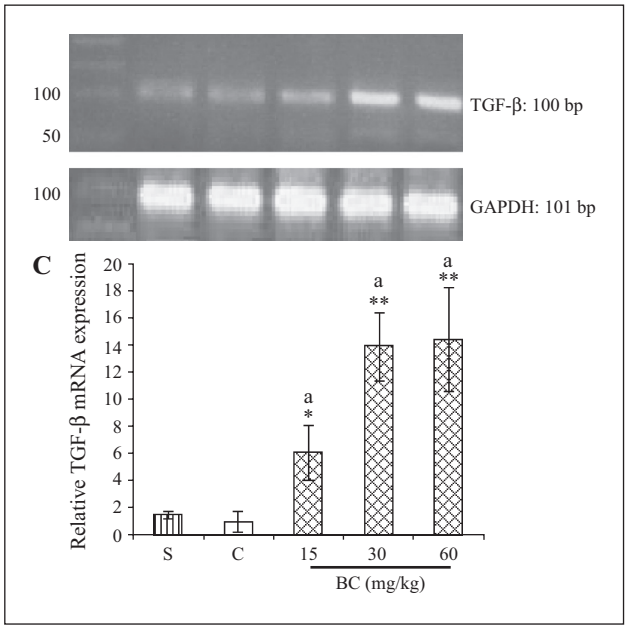

Figure 3 (Continued)

animals. These levels were significantly decreased in the β-carotene-pretreated rats compared with the control rats (*figures 4A,B*). In contrast, TGF-β plasma levels were significantly increased in β-carotene-pretreated groups compared with the control rats (*figures 4C*).

DISCUSSION

β-carotene has been reported to inhibit oxidant-mediated activation of inflammatory signaling and to suppress the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in gastric epithelial AGS cells infected with *Helicobacter pylori* [12]. Moreover, it has been shown that β-carotene decreases the gene expression of IL-1β and TNF-α in lipopolysaccharide-stimulated macrophages by suppressing redox-based nuclear factor-kβ activation [13]. Our findings also showed that the mRNA expression of pro-inflammatory cytokines, IL-1β and TNF-α, were decreased in β-carotene-pretreated rats in a dose-dependent manner. Our *in vivo* findings are consistent with previous *in vitro* reports [12, 13] that suggest that the reduction in mRNA and plasma levels of pro-inflammatory cytokines is a possible mechanism for the anti-inflammatory activity of β-carotene. Therefore, it can be concluded that the gastroprotective effect of β-carotene against I/R injury is partly mediated by a decrease the gene expression and plasma release of pro-inflammatory cytokines.

The tissue-protective effects of β-carotene have been shown to be largely associated with its antioxidant capacity [6, 7, 14, 15]. Recently, β-carotene has been shown to improve renal function following I/R injury by restoring the activity of the antioxidant enzymes (catalase, superoxide dismutase and glutathione peroxidase), and by inhibiting the peroxidation of lipids [6]. Furthermore, β-carotene has also been reported to attenuate indomethacin-induced gastric ulcers in rats through an increase in the levels of antioxidant enzymes and inhibition of lipid peroxidation [15]. In addition, in a rat model of hepatic I/R injury, supplementation with β-carotene

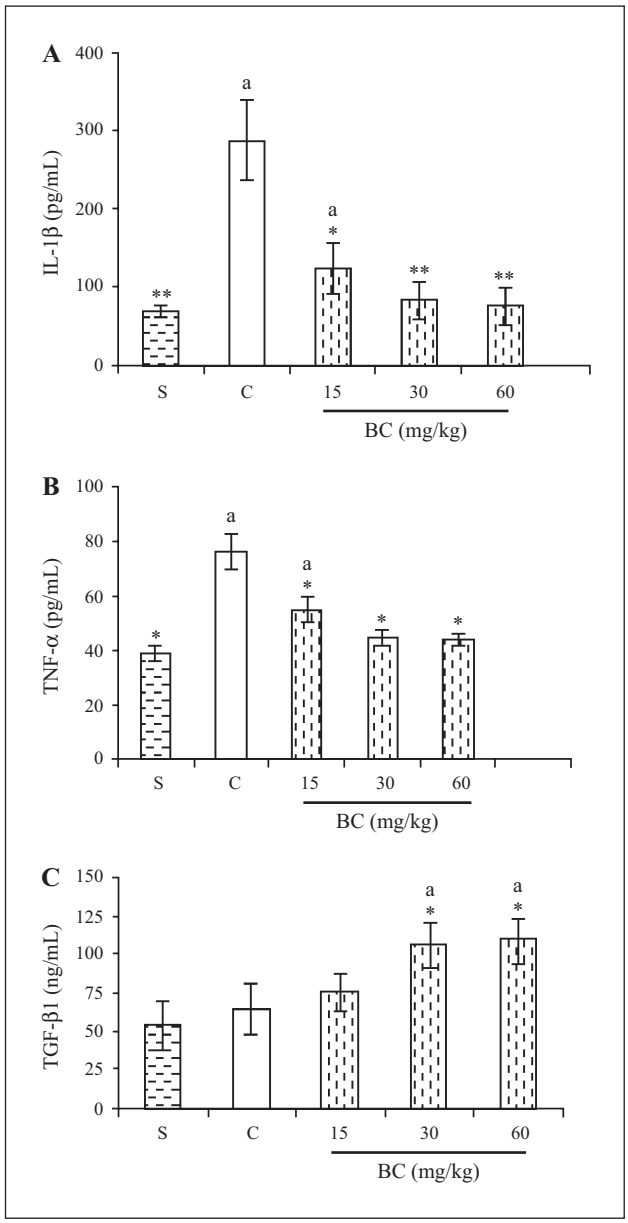

Figure 4
A graphic representation of the effect of pretreatment with β-carotene (15, 30 and 60 mg/kg, for five days) on plasma levels of cytokines. The plasma levels of (A) IL-1 β and (B) TNF-α were significantly decreased by pre-administration of β-carotene. While the plasma level of (C) TGF-β was significantly increased. S: sham, C: control, BC: β-carotene; *P<0.05,**P<0.01 *versus* the control group and [a]P<0.05 *versus* the sham group. Data are expressed as mean±S.E.M.

has been shown to increase the level of antioxidants [7]. Moreover, β-carotene has been demonstrated to protect the intestinal mucosa against the oxidative damage induced by methotrexate in rats by inhibiting the peroxidation of lipids, improving the activity of antioxidant enzymes and preventing the infiltration of neutrophils [14]. Taken together, these findings suggest that β-carotene has multiple beneficial roles as a gastroprotective agent, and might be responsible for the reduction in pro-inflammatory cytokine release and the increase in anti-inflammatory cytokines demonstrated in this study. Further, histopathological studies are needed to assess the source of these cytokines.

In this study, we have shown that β-carotene causes an increase in plasma levels and gene expression of the anti-inflammatory cytokine TGF-β. It has been shown that retinoic acid, which is a natural derivative of vitamin A, induces the expression of TGF-β2 [16]. β-carotene has been shown to inhibit the growth of cervical, intraepithelial neoplasia by inducing the anti-inflammatory cytokine, TGF-β [17]. In the present study, we have shown that mRNA expression and plasma levels of TGF-β were significantly increased by pretreatment with β-carotene. Therefore, the other possible mechanism by which β-carotene exerts its gastroprotective effect might be mediated by the up-regulating the anti-inflammatory cytokine, TGF-β.

Some previous literature has shown that β-carotene acts as a pro-oxidant under certain conditions such as high concentration and high tension of oxygen [18, 19]. A high-dose supplementation of β-carotene has been shown to impair mitochondrial function through a reduction in mitochondrial anti-oxidants [20, 21]. Hosseini *et al.* have shown that the protective effect of β-carotene on renal I/R injury was not affected by the dose [22]: they demonstrated that pretreatments with β-carotene at 30 and 100 mg/kg has similar protective effects on renal function in a rat model of renal I/R injury [22], whereas the present study showed that β-carotene protected the gastric mucosa on I/R injury in a dose-dependent manner. As shown in the Results, the total area of the mucosal lesions in group 3 (15 mg/kg of β-carotene) was higher than in group 4 (30 mg/kg of β-carotene) (*figures 1,2*). However, the findings also indicated that the protective effect of the highest dose of β-carotene (60 mg/kg) studied was similar to that seen with 30 mg/kg of β-carotene. Therefore, the optimal protective dose of β-carotene in gastric I/R injury was 30 mg/kg. Taken together, these results suggest that the protective effect of β-carotene at lower doses (30 mg/kg) may increase the safety of β-carotene in gastric I/R injury (as shown by the present study) and in renal I/R injury (as shown by Hosseini *et al.*) [22].

In conclusion, the present study, for the first time, has shown a gastroprotective effect of β-carotene in I.R injury. The findings of this study demonstrated that:

– pretreatment with β-carotene decreased the total area of acute gastric mucosal lesions induced by I/R, in a dose-dependent manner,

– the mRNA expression and plasma levels of IL-1β and TNF-α in β-carotene-pretreated rats were lower than in the control animals,

– the gene expression and plasma levels of the anti-inflammatory TGF-β cytokine were significantly increased by pretreatment with β-carotene.

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