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Protective Effect of Astaxanthin Produced by *Xanthophyllomyces dendrorhous* Mutant on Indomethacin-Induced Gastric Mucosal Injury in Rats

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Abstract Nonsteroidal anti-inflammatory drugs such as indomethacin induce severe gastric mucosal damage in humans and rodents. In the present study, the *in vivo* protective effect of astaxanthin on indomethacin-induced gastric lesions in rats was investigated. The test groups were injected with indomethacin (25 mg/kg) after the oral administration of astaxanthin (25 mg/kg) for 1, 2, and 3 days, while the control group was treated only with indomethacin. Thiobarbituric acid reactive substances in the gastric mucosa, as an index of lipid peroxidation, increased significantly after indomethacin administration and this increase was inhibited by oral administration of astaxanthin. In addition, pretreatment with astaxanthin resulted in a significant increase of the activities of superoxide dismutase (SOD), catalase, and glutathione peroxidase (GSH-px). Histologic examination clearly revealed acute gastric mucosal lesions induced by indomethacin in the stomach of the control group, but were not observed in that of the test group. These results indicate that astaxanthin activates SOD, catalase, and GSH-px, and removes the lipid peroxides and free radicals induced by indomethacin. It is evident that astaxanthin acts as a free radical quencher and antioxidant, and is an effective molecule in the remedy of gastric mucosal lesions.

Key words: Astaxanthin, indomethacin, gastric mucosal injury, gastritis, intragastric administration

Nonsteroidal anti-inflammatory drugs (NSAIDs) such as indomethacin have been widely used clinically as anti-inflammatory, analgesic agents, but it has been reported that gastrointestinal injury, one of the most serious side effects attributable to NSAIDs, may occur in patients treated with NSAIDs [5, 34]. Ulcerative lesions of the gastrointestinal tract are one of the major side effects of NSAIDs and are the major limitation to their use as anti-inflammatory drugs [3, 15].

Indomethacin, a noncorticosteroid drug with anti-inflammatory, antipyretic, and pain-relieving properties, is known to produce erosions, ulcerative lesions, and petechial bleeding in the mucosa of stomach as adverse effects [7, 19]. In rats, the oral intake of indomethacin induces ulcerations in the mucosa of the stomach [6]. Production of oxygen free radicals and lipid peroxidation plays a crucial role in the development of the gastric mucosal lesions induced by indomethacin [26, 27, 29, 30, 35, 36].

Carotenoids have been documented as providing several common biological functions such as photoprotection, antioxidant effects including singlet oxygen quenching, and immunomodulatory and anticancer activities, in both humans and rodents [4]. Approximately 600 carotenoids exist in nature, mostly contained in vegetables, some marine animals, and sea algae, and approximately 10% may serve as precursors of vitamin A. Lycopene, astaxanthin, canthaxanthin, and fucoxanthin are not precursors of vitamin A in mammals [22]. Astaxanthin, which belongs to the xanthophylls, inhibits urinary bladder carcinogenesis induced by *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine in mice [33], oral carcinogenesis induced by 4-nitrosoguanosine 1-oxide in rats [32], and colon carcinogenesis induced by azoxymethane in rats [31]. These results suggest that the protective effects of astaxanthin are independent of provitamin A activity and appear to be related to its effectiveness as an antioxidant and free radical scavenger.

Astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione) is widely distributed in nature and is the principal pigment in crustaceans, salmonoids, and many other organisms. But the biosynthesis of astaxanthin is limited to a few species of microorganisms such as *Xanthophyllomyces dendrorhous* and *Haematococcus pluvialis*. *X. dendrorhous* is a carotenoid-producing yeast which synthesizes astaxanthin as its main carotenoid [9]. *X. dendrorhous* has potential

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commercial value as a dietary source of natural astaxanthin. However, wild-type isolates of the yeast are uneconomical because of their low astaxanthin production and high production cost. In this report, an astaxanthin-overproducing mutant was developed and used [2, 11, 12, 17, 28].

Astaxanthin shows both a strong quenching effect against singlet oxygen and a strong scavenging effect against free radicals [13, 25]. Astaxanthin is also a powerful antioxidant and was shown to be most effective in stimulating immune defenses when different carotenoids were compared [10, 14, 25]. The antioxidant activity of astaxanthin has been reported to be approximately 10-fold stronger than that of other tested carotenoids, namely, zeaxanthin, lutein, canthaxanthin, and β-carotene, and 100-fold greater than that of α-tocopherol [13]. These effects are considered to be defense mechanisms for attacking reactive oxygen species (ROS). Furthermore, astaxanthin showed strong activity as an inhibitor of oxygen radical-mediated lipid peroxidation [18].

The aim of this study is to investigate the possibility of potential clinical application of astaxanthin from *X. dendrorhous*. This study investigated the *in vivo* protective effect of astaxanthin on indomethacin-induced gastric lesions in rats. The possible mechanisms of astaxanthin for gastroprotection were also investigated by measuring the amount of lipid peroxidation, and comparing the activities of enzymatic scavengers such as SOD, catalase, and GSH-px.

MATERIALS AND METHODS

Yeast Strains and Growth

Xanthophyllomyces dendrorhous ATCC 96594 was kindly provided by the Korea Research Institute of Bioscience and Biotechnology. The JH-1 astaxanthin-overproducing mutant was derived from *Xanthophyllomyces dendrorhous* ATCC 96594 by mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) [2, 11, 12, 17, 28]. Yeasts were maintained on slants of yeast extract/malt extract/peptone/glucose medium (YM broth, Difco) with 1.5% (v/v) agar (YM agar) and refrigerated at 4°C. Yeast strains were also stored in 40% glycerol/60% YM broth (v/v) at -70°C.

Astaxanthin Extraction and Analysis

For routine analyses of astaxanthin, the washed cell pellets of *X. dendrorhous* were mixed with DMSO preheated to 55°C, and then agitated for 1 min. The broken cells were thoroughly stirred in acetone, centrifuged, and the pigments in the supernatant were transferred to petroleum ether with the addition of 20% NaCl solution. Petroleum ether extracts were dried and concentrated by rotary evaporation. Astaxanthin was extracted from petroleum ether, and then quantitatively analyzed by HPLC. HPLC was used to identify the purified astaxanthin. HPLC was conducted on a Waters liquid chromatograph equipped with two 510 pumps and a 996 photodiode array detector. Astaxanthin was separated and analyzed using a LUNA C_{18} column (250×4.6 mm; 5 μm, *Phenomenex*) at 25°C at a flow rate of 1.0 ml/min. The mobile phase consisted of methanol (85%), dichloromethane (5%), acetonitrile (5.5%), and water (4.5%). Samples for HPLC analysis were dissolved in the mobile phase. Peaks were measured at a wavelength of 480 nm. Astaxanthin was identified according to its retention time and spectrum by photodiode array detection [37].

Animal Groups and Drug Preparation

Male Sprague-Dawley rats (230-250 g, 7 weeks old) were purchased from Daehan Biolink Co., Ltd. Rats were placed singly in cages with wire-net floors in a controlled room (temperature 22-24°C, humidity 70-75%, lighting regimen of 12 h light and 12 h dark) and were fed a normal laboratory diet. Rats were deprived of food for 24 h before experimentation but allowed free access to tap water throughout.

Gastric mucosal damage was induced by a single oral dose of indomethacin. Indomethacin was dissolved in 5% sodium bicarbonate and was administered intragastrically to rats in a dose of 25 mg/kg in a volume of 0.5 ml/100 g body weight. Astaxanthin (25 mg/kg) was dissolved in a medium chain triglyceride (MCT) solution immediately before use and was administered by oral treatment. The animals were divided into six groups (n=6 rats per group). The normal group received 5% sodium bicarbonate orally in a volume of 0.5 ml/100 g body weight. Control 1 was treated only with indomethacin. Control 2 was treated with indomethacin after oral intake of a medium chain triglyceride (MCT) solution for 3 days. Test 1, test 2, and test 3 were pretreated with astaxanthin for 1, 2, and 3 days, respectively, and received indomethacin at 1 h after the last oral administration of astaxanthin.

The animals were killed under deep ether anesthesia at 4 h after indomethacin treatment. The rat's stomach was promptly excised, weighed, and chilled in ice-cold 0.9% NaCl. After washing with 0.9% NaCl, the mucosa was homogenized in 50 mM potassium phosphate buffer at pH 7.5. Mitochondria and cytosol fractions were prepared according to the method of Hogeboom [8]. The quantitative analysis of protein was measured by the Bradford protein assay.

Determination of Lipid Peroxidation

The level of thiobarbituric acid (TBA) reactants in the gastric mucosa was measured according to the modified method of Ohkawa *et al.* [23]. The mucosa was homogenized in 50 mM potassium phosphate buffer at pH 7.5. The homogenate was supplemented with 8.1% sodium dodecyl sulfate, 20% acetic acid (pH 3.5), and 0.8% TBA, and

boiled at 95°C for 1 h. After cooling with tap water, the reactants were supplemented with *n*-butanol and pyridine (15:1 v/v), shaken vigorously for 1 min, and centrifuged for 10 min at 4,000 rpm. Absorbance was measured at 532 nm and the results were expressed as nmol TBA/g of tissue weight. The level of TBA-reactive substances was expressed in terms of nmol of malondialdehyde (MDA).

Determination of SOD Activity

SOD activity in the gastric mucosa of rats was determined according to the method of McCord and Fridovich [21]. The standard assay was performed in 3 ml of 50 mM potassium phosphate buffer at pH 7.8 containing 0.1 mM EDTA in a cuvette thermostated at 25°C. The reaction mixture contained 0.1 mM ferricytochrome *c*, 0.1 mM xanthine, and sufficient xanthine oxidase to produce a rate of reduction of ferricytochrome *c* at 550 nm of 0.025 absorbance unit per min. Tissue homogenate was mixed with a reaction mixture (50 mM potassium phosphate buffer, pH 7.8, containing 0.1 mM EDTA, 0.1 mM ferricytochrome *c*, 0.1 mM xanthine). Kinetic spectrophotometric analysis was started when xanthine oxidase was added. The amount of SOD required to inhibit the rate of reduction of cytochrome *c* by 50% was defined as 1 unit of activity. Absorbance was measured at 550 nm and the results were expressed as units/mg of protein.

Determination of Catalase Activity

Catalase activity in the gastric mucosa of rats was determined according to the method of Aebi [1]. The standard assay was performed in 3 ml of 50 mM potassium phosphate buffer (1.9 ml) at pH 7.0 containing 10 mM H_2O_2 (1 ml), and tissue homogenate (100 ml). The amount of catalase required to decompose 1.0 μmole of H_2O_2 per min at pH 7.0 at 25°C was defined as 1 unit of activity. Absorbance was measured at 240 nm for 2 min and the results were expressed as units/mg of protein.

Determination of Glutathione Peroxidase Activity

GSH-px activity in the gastric mucosa of rats was determined by modifying the method of Lawrence and Burk [16]. The reaction mixture consisted of glutathione peroxidase assay buffer (50 mM potassium phosphate buffer pH at 8.0, 0.5 mM EDTA) and NADPH assay reagent (5 mM NADPH, 42 mM GSH, 10 unit/ml glutathione reductase). A sample of supernatant fluid, which included homogenate solution and 50 mM potassium phosphate buffer at pH 7.5, was prepared by centrifugation at 1,000 ×*g* for 10 min at 4°C. To the cuvette, 900 μl of glutathione peroxidase assay buffer, 50 μl of NADPH assay reagent, and 50 μl of sample were added. Subsequently, all of them were mixed together by inversion. The reaction was started by the addition of 10 μl of 30 mM tert-butyl hydroperoxide or 80% Cumene hydroperoxide. Absorbance was recorded as follows; wavelength: 340 nm; initial delay: 15 sec; interval: 10 sec; number of readings: 6. The level of GSH is expressed in terms of μmole/min/mg of protein.

Histopathology

Murine stomach tissues were fixed in 10% neutral formalin and embedded in paraffin. Then, 4-μm-thick sections were prepared and stained with hematoxylin and eosin by standard procedures.

Statistical Analysis

All results are expressed as means±S.E.M. Duncan's multiple range test was performed to compare the normal group, control groups, and test groups. Statistical significance was defined by ***P*** values of less than 0.05.

RESULTS

Effect of Astaxanthin on Changes in Amount of Lipid Peroxidation

The concentration of MDA in the gastric mucosa, as an index of lipid peroxidation, was measured at 4 h after indomethacin administration. The MDA concentration increased significantly to 18.08±0.98 and 18.62±0.79 nmole/g of tissue in the control 1 and control 2 groups, respectively, compared with the concentration (10.83±0.84 nmole/g of tissue) in the normal group ($P<0.01$). The test 1, test 2, and test 3 groups had concentrations of 16.22±0.48, 15.50±0.54, and 10.30±0.51 nmole/g of tissue, respectively. These test groups showed significant decreases in lipid peroxidation compared with levels in the control 1 and control 2 groups ($P<0.001$) (Fig. 1).

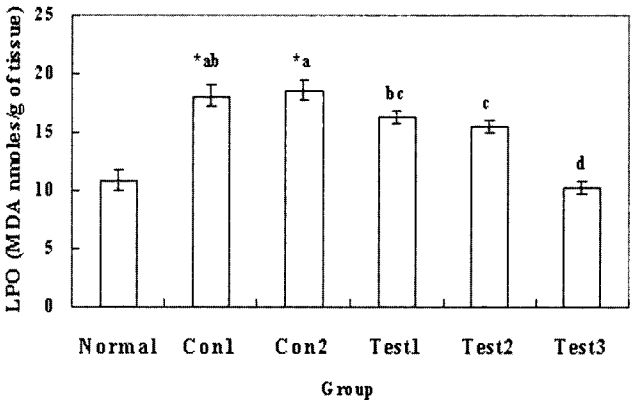

Fig. 1. Effects of pretreatment with astaxanthin (25 mg/kg for 1, 2, and 3 days, respectively) on LPO in gastric lesions induced by indomethacin in rats.
The MDA concentration increased significantly in the control 1 and control 2 groups compared with the concentration in the normal group (*$P<0.01$). The test groups had significantly decreased MDA concentrations compared with levels in the control groups ($^{a,b,c,d}P<0.001$). Results are expressed as means±S.E.M., n=6.

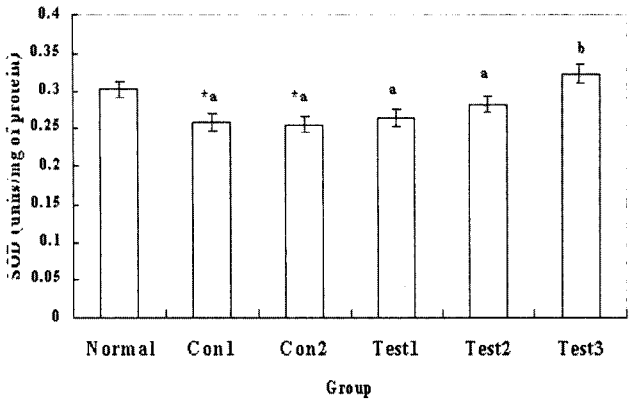

Fig. 2. Effects of pretreatment with astaxanthin (25 mg/kg for 1 2, and 3 days, respectively) on SOD activity in gastric lesions induced by indomethacin in rats.
SOD activity decreased significantly in the control 1 and control 2 groups compared with activity in the normal group (*P<0.01). The test groups had significantly increased SOD activity compared with activity in the control groups ($^{a,b}P$<0.001). Results are expressed as means±S.E.M., n=6.

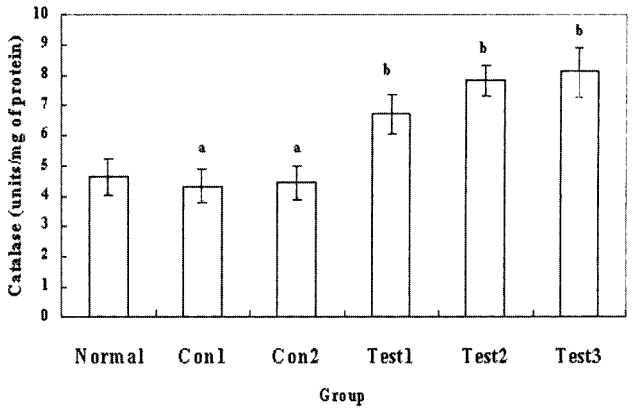

Fig. 3. Effects of pretreatment with astaxanthin (25 mg/kg for 1, 2, and 3 days, respectively) on catalase activity in gastric lesions induced by indomethacin in rats.
No significant difference in catalase activity was evident between the normal, control 1, and control 2 groups. The test groups showed significant increases in catalase activity compared with activity in the control groups ($^{a,b}P$<0.01). Results are expressed as means±S.E.M., n=6.

Effect of Astaxanthin on SOD Activity

SOD activity in the gastric mucosa decreased significantly to 0.258±0.011 and 0.255±0.011 units/mg of protein in the control 1 and control 2 groups, respectively, at 4 h after indomethacin administration, compared with activity (0.302± 0.010) in the normal group (P<0.01). The test 1 and test 2 groups showed 0.264±0.011 and 0.282±0.011 units/mg of protein in SOD activity, respectively. These values of the test groups showed a small increase in activity of SOD compared with values in the control 1 and control 2 groups (P<0.001). However, SOD activity in the gastric mucosa increased significantly to 0.322± 0.012 units/mg of protein in the test 3 group compared with activity in the control 1 and control 2 groups (P<0.001) (Fig. 2).

Effect of Astaxanthin on Catalase Activity

Catalase activity in the gastric mucosa was 4.63±0.57 units/mg of protein in the normal group, 4.34±0.54 units/mg of protein in the control 1 group, and 4.44±0.55 units/mg of protein in the control 2 group. The values of the control groups showed a small decrease in the activity of catalase compared with the value in the normal group. However, no significant difference in catalase activity was found between either the normal, control 1, or control 2 groups. The test 1, test 2, and test 3 groups had 6.72±0.67, 7.82± 0.51, and 8.10±0.83 units/mg of protein in catalase activity, respectively. These test groups showed significant increases in the activity of catalase compared with activity in the control 1 and control 2 groups (P<0.01) (Fig. 3).

Effect of Astaxanthin on GSH-px Activity

After intragastric administration of indomethacin, GSH-px activity in the control 1 and control 2 groups reduced significantly to 8.48±0.55 and 8.24±0.71 μmole/min/mg of protein, respectively, compared with activity (12.74± 0.52) in the normal group (P<0.01). The test 1, test 2, and test 3 groups had 12.71±0.87, 12.75±0.50, and 12.63± 0.64 μmole/min/mg of protein in GSH-px activity, respectively. These results showed that the activity of GSH-px in these test groups increased significantly compared with activity in the control 1 and control 2 groups (P<0.001) (Fig. 4).

Effect of Astaxanthin on Gastric Lesions Induced by Indomethacin

The administration of indomethacin induced gastric hemorrhagic lesions, affecting mostly the glandular portion

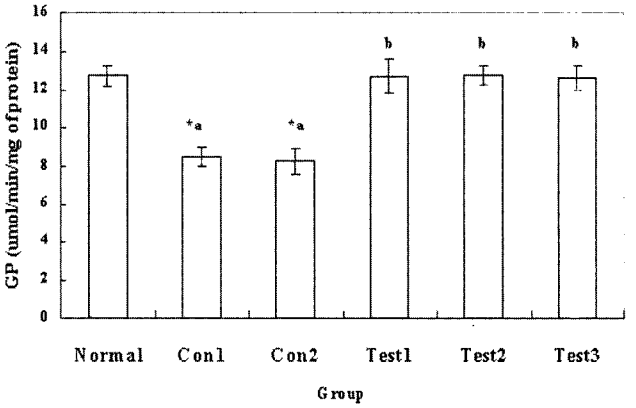

Fig. 4. Effects of pretreatment with astaxanthin (25 mg/kg for 1, 2, and 3 days, respectively) on GSH-px activity in gastric lesions induced by indomethacin in rats.
GSH-px activity reduced significantly in the control 1 and control 2 groups compared with activity in the normal group (*P<0.01). The test groups showed significant increases in GSH-px activity compared with activity in the control groups ($^{a,b}P$<0.001). Results are expressed as means±S.E.M., n=6.

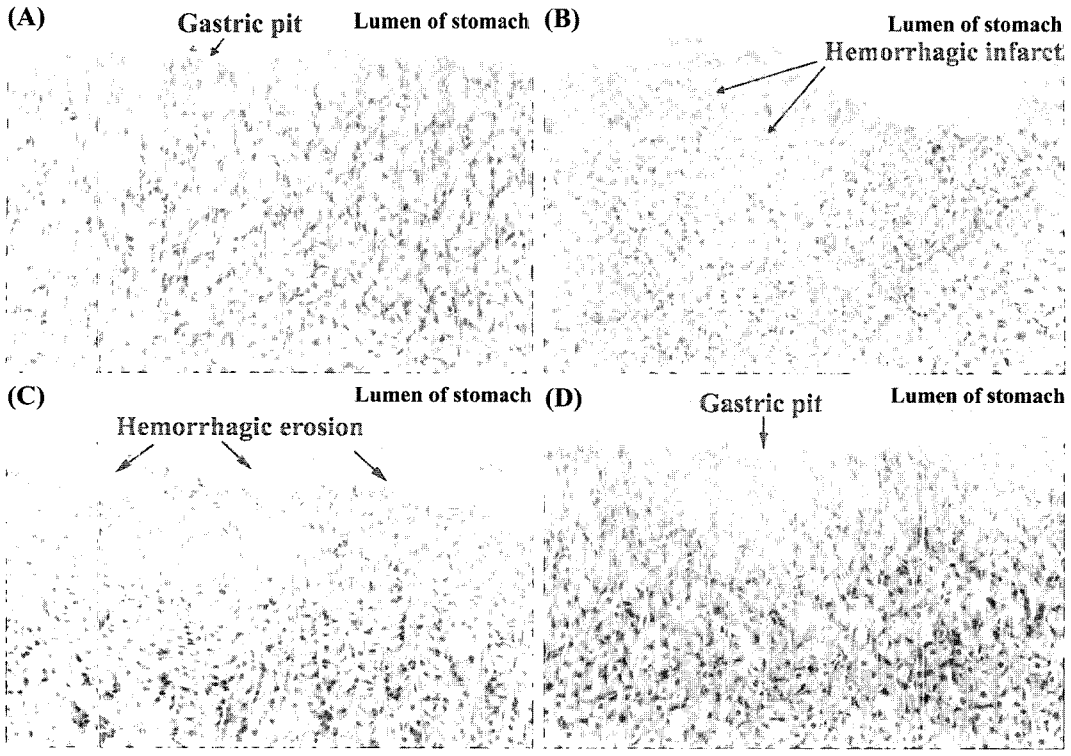

Fig. 5. Histology of the hemorrhagic infarct and erosion of mucosa in rats (hematoxylin and eosin ×200).
(A) Normal fundic mucosa from a normal rat. (B) The hemorrhagic infarct of mucosa in a stomach treated with indomethacin. The surface mucrus cells were necrotic, and gastric pits disappeared in the hemorrhagic infarct. (C) The hemorrhagic erosion of mucosa in a stomach treated with indomethacin. Hemorrhagic necrosis and diffused necrosis of villous ridges were present, and the villous tip of the mucosa was completely destroyed. (D) The mucosa in a rat treated with astaxanthin 25 mg/kg for 3 days.

of the mucosa. The lesions were long and thin in appearance, black in coloration, and were usually seen along the crests of the sides of rugal folds. The total area of gastric erosions was significantly larger in the indomethacin-treated group than the normal group.

In this study, the normal group showed normal fundic mucosa (Figs. 5A, 6A). The effects induced by indomethacin, such as inflammatory cell infiltration, mucosa hemorrhagic infarct, mucosa cell necrosis, mucosa hemorrhagic erosion, and gastric pit disappearances, were observed in the stomachs of the control 1 group (Figs. 5B, 5C, 6B). Congestion and gland atrophy were also observed in the stomachs of the control 1 group (Fig. 7), and lymphoid follicles were only observed in the stomachs of that group (Fig. 8). Histologic examination revealed that the indomethacin-induced lesions in the control 1 group penetrated deeply into the mucosa. Hemorrhagic necrosis and diffused necrosis of villous ridges were present, and the villous tip of the mucosa was completely destroyed (Figs. 5B, 5C).

However, the test 3 group showed nearly normal morphology. Inflammatory cell infiltration, mucosa hemorrhagic infarct, mucosa cell necrosis, congestion, and mucosa hemorrhagic erosion occurred far less, and gastric pit disappearance was not observed at all in the stomachs of the test 3 group (Figs. 5D, 6C). Lymphoid follicles were not observed in the stomachs of the test 3 group. Less gland atrophy developed in the test 3 group compared to the control 1 group. Treatment with astaxanthin of 25 mg/kg for 3 days reduced the depth and severity of lesions (Fig. 5). Mild to moderate villous atrophy was noted, and severe necrotic changes in mucus ridges were not observed. Histologic examination clearly indicated that pretreatment with astaxanthin before indomethacin administration effectively prevented gastric injury.

DISCUSSION

The most acute adverse effect of NSAIDs such as indomethacin, in terms of both frequency and clinical impact, is the ulceration that can be observed in the gastrointestinal tract [3, 5, 34]. Indomethacin comprises polar lipids that have a high affinity for the lipophilic areas of cell membranes, where their polar groups trigger membrane disruption, with loss of structural phospholipids and membrane proteins. This also leads to reduced hydrophobicity of the mucosal coat adherent to the mucosal cell surface. Such loss of hydrophobicity facilitates the entry of water-

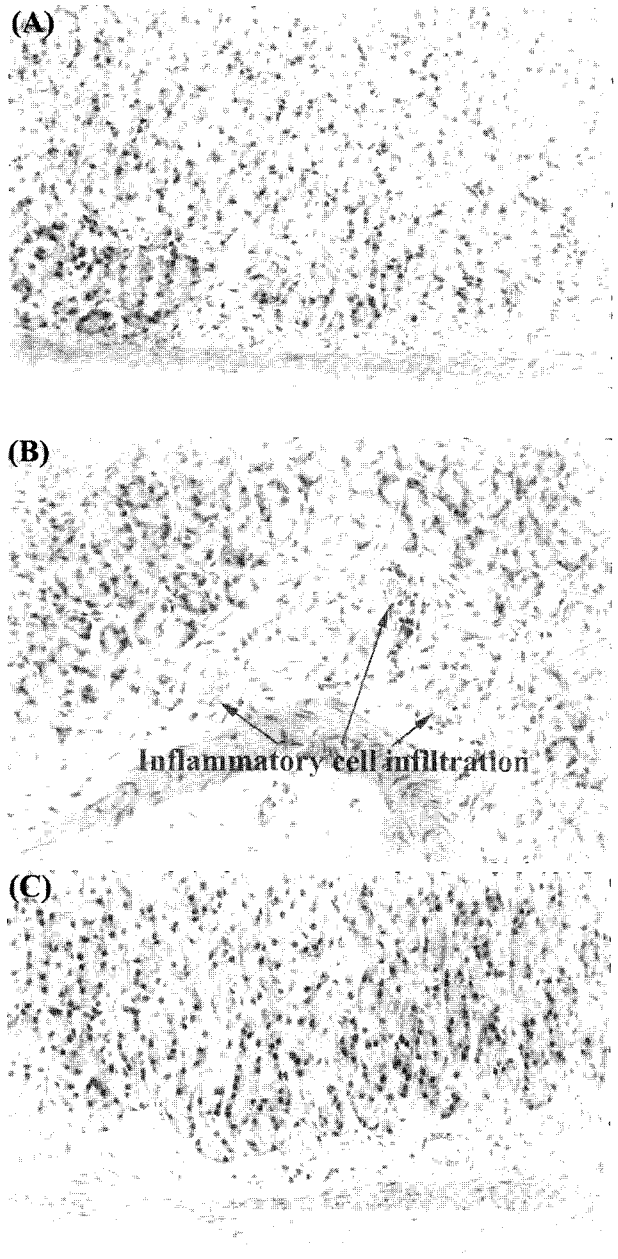

Fig. 6. Histology of inflammatory cell infiltration within the mucosa in rats (hematoxylin and eosin ×100).
(A) Normal fundic mucosa from an untreated control rat. (B) Tissue from an indomethacin-treated rat with a large amount of acute inflammatory cell infiltration within the mucosa and along the laminar muscular mucosa. (C) Less inflammation (small amount of inflammatory cell infiltration) in a rat treated with astaxanthin 25 mg/kg for 3 days.

soluble agents of injury, e.g. acid, pepsin, bile salts, etc., which cause lipid peroxidation and also alter membrane fluidity [20]. Intragastric administration of indomethacin in rats induced hemorrhagic lesions in the glandular stomachs by means of lipid peroxidation and the generation of oxygen free radicals [6, 19, 29]. In the present study, the

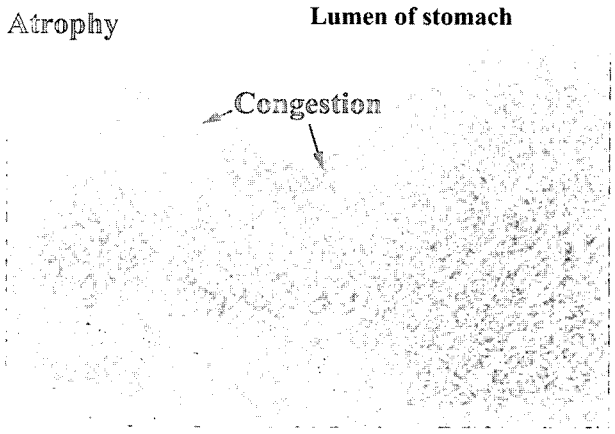

Fig. 7. Histology of gland atrophy and the congestion of mucosa in an indomethacin-treated rat.

in vivo protective effect of astaxanthin on indomethacin-induced gastric mucosal lesions in rats was examined. Peroxidation of lipids and changes in the activities of healing-related enzymes such as SOD, catalase, and GSH-px were also monitored.

As an experimental model in this study, astaxanthin provided remarkably potent protection against lipid peroxidation. TBA-reactive substances in the gastric mucosa were as an index of lipid peroxidation, which increased significantly after indomethacin administration in the control 1 group, and this increase was inhibited in test groups by oral administration of astaxanthin for different days. This finding suggests that astaxanthin significantly inhibits indomethacin-induced lipid peroxidation.

Enzymes such as SOD, catalase, and GSH-px provide defenses against oxidative tissue damage of the gastric mucosa occurring after the administration of indomethacin. The activities of SOD, catalase, and GSH-px were significantly inhibited by indomethacin administration in the control 1 group, which indicated that inhibition of these enzymatic activities was, at least in part, responsible for oxidative tissue damage of gastric mucosa occurring after the administration of indomethacin.

SOD is a family of predominantly intracellular metalloproteins that catalyzes the dismutation of O_2^- to H_2O_2 in gastric mucosal cell [21, 26]. In the present study, the activity of SOD in the gastric mucosa decreased significantly after indomethacin administration in the control 1 group compared with the activity in the normal group. Pretreatment with astaxanthin for different days before indomethacin administration significantly increased SOD activity in the gastric mucosa compared with the activity in the control 1 group.

Catalase is a hemoprotein which catalyzes the decomposition of H_2O_2 to water in the gastric mucosal cell [1, 26]. The present study showed that the activity of catalase in the gastric mucosa also increased significantly

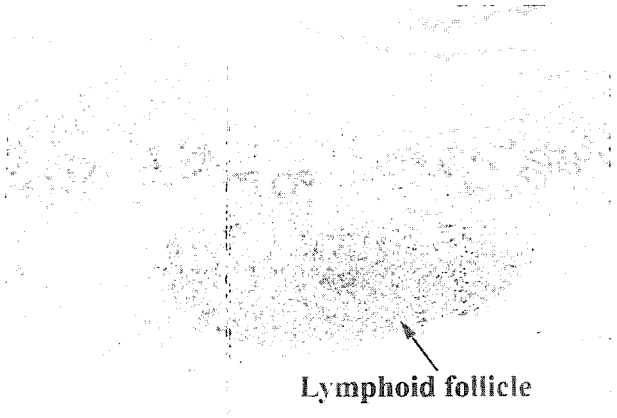

Fig. 8. Histology of the lymphoid follicle of the mucosa in an indomethacin-treated rat.

in test groups pretreated with astaxanthin over several days before the administration of indomethacin.

GSH-px is an important enzyme that plays a role in the elimination of H_2O_2 and lipid hydroperoxides in the gastric mucosal cell [26]. The antioxidant activity of GSH-px is coupled with the oxidation of reduced glutathione that can subsequently be reduced by glutathione reductase with NADPH as a reducing agent. Thus, inhibition of this enzyme may result in the accumulation of H_2O_2 with subsequent oxidation of lipids. The present study also revealed that astaxanthin administration over several days resulted in a significant increase of GSH-px activity compared with levels in the control 1 group.

From these results, it can be concluded that astaxanthin might protect rat gastric mucosa by its ability to increase the activities of free radical scavenging enzymes such as SOD, catalase, and GSH-px in the mucosa.

Histologic examination clearly revealed that the indomethacin-induced gastric hemorrhagic lesions were prevented by pretreatment with astaxanthin. In this report, the side effects induced by indomethacin, such as inflammatory cell infiltration, mucosa hemorrhagic infarct, mucosa cell necrosis, congestion, mucosa hemorrhagic erosion, and gastric pit disappearance, were observed in the stomachs of the control 1 group, while these kinds of symptoms were either subsided or not observed at all in the stomachs of the test 3 group.

The antioxidant properties of astaxanthin have been investigated in many experiments in recent years. In other experimental models, astaxanthin has been established as being a powerful antioxidant *in vitro* [13, 25], and has been shown to prevent oral carcinogenesis in an experimental rat model [32]. It is now well known that other carotenoids enhance the immune defenses, and in comparative studies, astaxanthin was shown to be the most effective [10, 24].

In conclusion, it was found in this study that astaxanthin activates SOD, catalase, and GSH-px, and removes the lipid peroxides and free radicals induced by indomethacin. It is evident that astaxanthin acts as a free radical quencher and antioxidant, and is an effective molecule in the remedy of gastric mucosal lesions.

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