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Therapeutic Effect of Astaxanthin Isolated from *Xanthophyllomyces dendrorhous* Mutant Against Naproxen-Induced Gastric Antral Ulceration in Rats

KIM, JEONG-HWAN[1], SEUNG-WOOK KIM[2], CHEOL-WON YUN[1], AND HYO-IHL CHANG[1]*

[1]*Department of Biotechnology, School of Life Sciences and Biotechnology, Korea University, Seoul 136-701, Korea*
[2]*Department of Chemical and Biological Engineering, Korea University, Seoul 136-701, Korea*

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Abstract Frequently used for humans as a nonsteroidal anti-inflammatory drug, naproxen has been known to induce ulcerative gastric lesions. The present study was undertaken to investigate the *in vivo* therapeutic effect of astaxanthin, isolated from a *Xanthophyllomyces dendrorhous* mutant, against naproxen-induced gastric antral ulceration in rats. The rats were treated with three doses of astaxanthin [1, 5, and 25 mg/kg body weight (B.W.), respectively] once daily for 2 weeks after pretreatment of 80 mg of naproxen/kg B.W. twice daily for 3 days, while the control rats received only 80 mg of naproxen/kg B.W. twice daily for 3 days. The oral administration of astaxanthin (1, 5, and 25 mg/kg B.W.) showed a curative effect against naproxen (80 mg/kg B.W.)-induced gastric antral ulcer and reduced the elevated lipid peroxide level in gastric mucosa. In addition, astaxanthin treatment resulted in significant increase in the activities of radical scavenging enzymes such as superoxide dismutase, catalase, and glutathione peroxidase. A histologic examination clearly proved that acute gastric mucosal lesion induced by naproxen nearly disappeared after the astaxanthin treatment. These results suggest that astaxanthin eliminated the lipid peroxides and free radicals induced by naproxen and may be a potential candidate for remedy of gastric ulceration.

Key words: Anti-ulcer drug, astaxanthin, gastric antral ulceration, orogastric administration, *Xanthophyllomyces dendrorhous*

Many nonsteroidal anti-inflammatory drugs (NSAIDs), including naproxen, have been widely used clinically as anti-inflammatory, analgesic agents. However, these compounds induce severe gastric mucosal lesions as one of the major side effects, and it is the major limitation to their use as anti-inflammatory drugs [4, 6, 19, 39]. Naproxen is a noncorticosteroid drug with anti-inflammatory, antipyretic, and pain-relieving properties, which is known to produce erosions, antral ulceration, and petechial bleeding in the mucosa of stomach as adverse effects. In the present study, naproxen was chosen as the ulceration-causing NSAID in rats, because the naproxen-induced gastric antral ulcer model represents the human situation the best, where NSAID-induced gastric ulceration occurs mainly in the gastric antrum [4, 8, 9, 19, 23, 24, 32, 35, 39]. The oral intake of naproxen causes antral ulceration in rats, and the production of oxygen free radicals and lipid peroxidation play a crucial role in the development of naproxen-induced gastric antral ulceration [8, 31, 35, 40].

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. However, the biosynthesis of astaxanthin has been established only in a few species of microorganisms such as *Xanthophyllomyces dendrorhous* and *Haematococcus pluvialis* [2, 11]. *X. dendrorhous* is a carotenoid-producing yeast, which synthesizes astaxanthin as its main carotenoid [11]. However, wild-type *X. dendrorhous* is uneconomical, because of its low astaxanthin content and high production cost. Therefore, an astaxanthin-overproducing *X. dendrorhous* mutant has been developed in our laboratory [14].

Astaxanthin has important metabolic functions in animals, including it's conversion to vitamin A [5], enhancement of immune response [12, 29], and protection against diseases such as cancer by scavenging oxygen radicals [13, 36, 37, 38]. The antioxidant activity of astaxanthin has been reported to be approximately 10 times stronger than that of other carotenoids tested, including zeaxanthin, lutein, canthaxanthin, and β-carotene, and 100 times greater than that of α-tocopherol [17, 26, 27]. These effects are considered to be defense mechanisms against the attack of reactive oxygen species. Astaxanthin also has strong activity as an inhibitor of oxygen radical-mediated lipid peroxidation [15].

**Corresponding author*
Phone: 82-2-3290-3421; Fax: 82-923-9923;
E-mail: hichang@korea.ac.kr

The aim of this study was to determine the therapeutic effect of astaxanthin against naproxen-induced gastric antral ulceration in rats by measuring the amount of lipid peroxidation and the activities of enzymatic scavengers such as superoxide dismutase, catalase, and glutathione peroxidase.

MATERIALS AND METHODS

Yeast Strains and Astaxanthin Extraction

X. dendrorhous ATCC 96594 was provided by Korea Research Institute of Bioscience and Biotechnology. The astaxanthin-overproducing mutant JH-1 was derived from *X. dendrorhous* ATCC 96594 by mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine [3, 14, 16, 21, 34]. For routine analysis of astaxanthin, the washed cell pellets were mixed with dimethyl sulfoxide preheated at 55°C and then agitated for 1 min. The broken cells were thoroughly stirred in acetone and 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 quantitatively analyzed by HPLC (Waters Co., U.S.A.), 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 and HPLC-grade astaxanthin (Sigma Co., U.S.A.) as standards [33]. The results of HPLC indicated that the purity of astaxanthin was more than 95% [14].

Chemicals

Naproxen (sodium salt) was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Naproxen was dissolved in distilled water and subsequently administered by orogastric gavage, with an appropriate feeding needle. The dose volume was 5 ml/kg B.W. Astaxanthin was dissolved in medium-chain triglyceride solution immediately before use and administered intragastrically to rats in a volume of 5 ml/kg B.W.

Animal Treatment

Sprage-Dawley male rats (230–250 g, 7 weeks old) were purchased from Daehan Biolink Co., Ltd. Rats were singly placed 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. Typically, rats were fasted for 18 h prior to studies. Following the first dose of naproxen, rats were provided with food for the remainder of the study. Rats were also allowed tap water throughout the study period.

To evaluate the effect of astaxanthin, the rats were divided into six groups (n=8 rats per group). The untreated normal rats received distilled water twice daily (at 07.00 h and 17.00 h) for 3 days, in comparable volume by oral route. The control rats received only 80 mg of naproxen/kg B.W. twice daily (at 07.00 h and 17.00 h) for 3 days. Each of the remaining four groups was treated with a medium-chain triglyceride solution (vehicle) and three doses of astaxanthin (1, 5, and 25 mg/kg B.W., respectively) once daily for 2 weeks after pretreatment of 80 mg of naproxen/kg B.W. twice daily (at 7.00 h and 17.00 h) for 3 days. All the rats were killed under deep ether anesthesia 4 h after the last oral administration of naproxen/astaxanthin. The rat stomachs were 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 [10]. The quantitative analysis of protein was carried out by Bradford protein assay [7].

Measurement of Lipid Peroxidation

Lipid peroxidation was determined by measuring the concentration of malondialdehyde in the gastric mucosa according to the modified method of Ohkawa *et al.* [28]. The stomach 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 mixed with *n*-butanol and pyridine (15:1 v/v), shaken vigorously for 1 min, and centrifuged for 10 min at 3,500 ×*g*. Absorbance was measured at 532 nm. Lipid peroxidation was calculated from the standard curve using the malondialdehyde tetrabutylammonium salt and expressed as the concentration of nmol of malondialdehyde per g of tissue weight.

Measurement of Superoxide Dismutase Activity

The activity of superoxide dismutase in gastric mucosa of rats was determined according to the method of McCord and Fridovich [25]. 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 reduction rate of ferricytochrome *c* at 550 nm of 0.025 absorbance unit per min. Tissue homogenate was mixed with the 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 by adding xanthine oxidase at 550 nm. Under these conditions, the amount of superoxide dismutase required to inhibit the reduction rate of cytochrome *c* by 50% was defined as 1 unit of activity. The results were expressed as units/mg of protein.

Measurement of Catalase Activity

The activity of catalase in 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 at pH 7.0 (1.9 ml) containing 10 mM H_2O_2 (1 ml) and tissue homogenate (100 μl). Under these conditions, the amount of catalase required to decompose 1.0 μmol of H_2O_2 per min at pH 7.0 and 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.

Measurement of Glutathione Peroxidase Activity

The activity of glutathione peroxidase in the gastric mucosa of rats was determined by a modified method of Lawrence and Burk [20]. The reaction mixture consisted of glutathione peroxidase assay buffer [50 mM potassium phosphate buffer (pH 8.0) and 0.5 mM EDTA] and NADPH assay reagent (5 mM NADPH, 42 mM reduced glutathione, 10 units/ml glutathione reductase). A supernatant of homogenate in 50 mM potassium phosphate buffer at pH 7.5 was prepared by centrifuging it at 1,000 ×*g* for 10 min at 4°C. Subsequently, 900 μl of glutathione peroxidase assay buffer, 50 μl of NADPH assay reagent, and 50 μl of the sample were added to the cuvette, and the contents were mixed by inversion. The reaction was started by adding 10 μl of 30 mM *tert*-butyl hydroperoxide or 80% cumene hydroperoxide. Absorbance was recorded by the following program: Wavelength, 340 nm; Initial delay, 15 s; Interval, 10 s; Number of readings, 6. The activity of the enzyme was the sum of data obtained by using 30 mM *tert*-butyl hydroperoxide and 80% cumene hydroperoxide. The level of glutathione was expressed in terms of μmol/min/mg of protein.

Histopathology

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

Statistical Analysis

All values were represented as means±S.E.M. Data were analyzed by ANOVA according to General Linear Model procedure. The means were compared by Tukey's Studentized Range (HSD) test to detect significant differences at $P<0.05$. All statistical procedures were performed with the SAS® software package (Release 8.02, 2001).

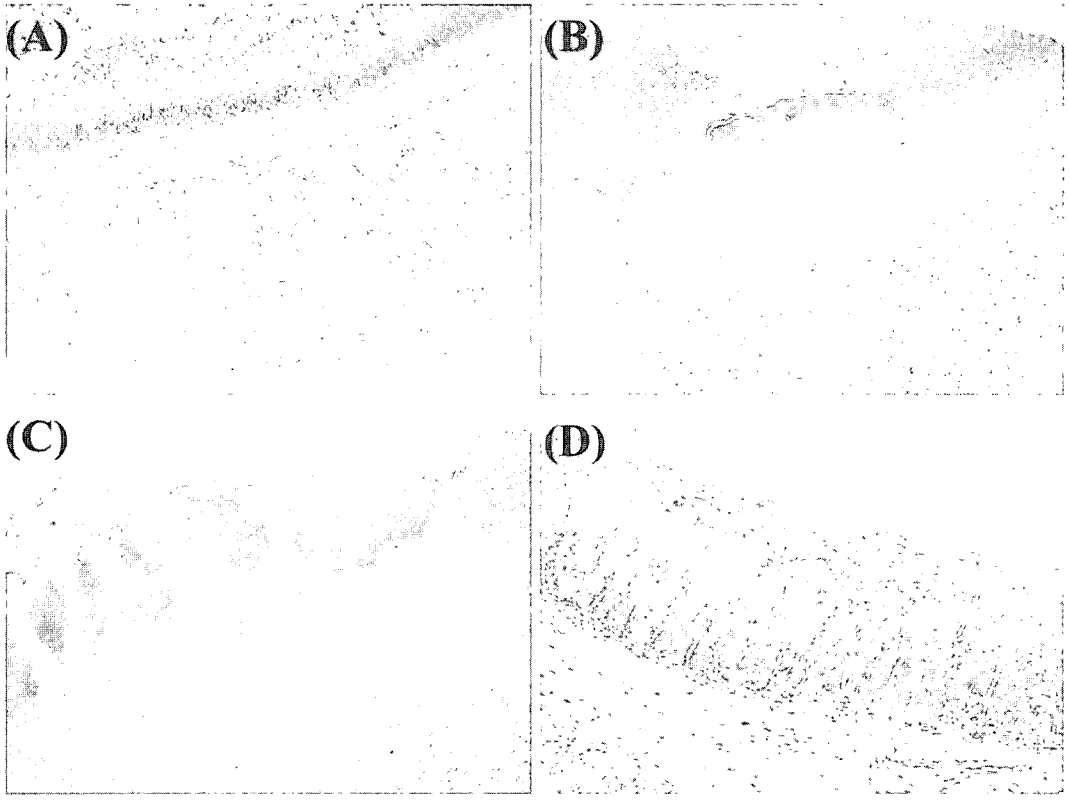

Fig. 1. Therapeutic effect of astaxanthin on naproxen-induced gastric antral ulceration in rats.
The rats received 25 mg of astaxanthin/kg B.W. once daily for 2 weeks after pretreatment with 80 mg of naproxen/kg B.W. twice daily for 3 days. (A) Normal gastric antrum from the untreated normal rat. (B) Gastric antral ulcer in the naproxen-treated rat. A gastric ulcer is clearly visible in the gastric antrum. (C) Deep erosion (ulceration) of gastric antrum in the naproxen-treated rat. Gastric lesion penetrated deeply into the muscularis mucosa and showed a large amount of acute inflammatory cell infiltration within the mucosa and along the muscularis mucosa. Bacteria infection was shown in this lesion. (D) Gastric antrum in the astaxanthin-treated rat. Treatment with 25 mg of astaxanthin/kg B.W. for 2 weeks healed inflammation and gastric antral ulcer.

RESULTS

Effect of Astaxanthin Against Naproxen-Induced Gastric Antral Ulceration in Rats

Ulceration was judged macroscopically by clear depth of penetration into the gastric mucosal surface. Superficial or deep erosions, bleeding, and antral ulcers were observed in rats that received 80 mg of naproxen/kg B.W. for 3 days. However, treatment with 25 mg of astaxanthin/kg B.W. for 2 weeks reduced the depth and severity of naproxen-induced gastric antral ulcer (Fig. 1).

The concentration of malondialdehyde in the control (naproxen, 80 mg/kg) and the vehicle (astaxanthin, 0 mg/kg)-treated rats was increased to 19.89±1.77 and 19.52±1.80 nmol/g of tissue, respectively, whereas the concentration of malondialdehyde in the untreated normal rats remained at 10.87±0.47 nmol/g of tissue ($^{+}P<0.05$). This increase in the concentration of malondialdehyde was reduced in a dose-dependent manner in all astaxanthin-treated rats. Specifically, treatment with 5 and 25 mg of astaxanthin/kg B.W. for 2 weeks significantly ($^{*}P<0.05$, $^{**}P<0.01$) decreased the concentration of malondialdehyde, compared to that in the control rats (Fig. 2).

The activity of superoxide dismutase in the control and the vehicle-treated rats was reduced to 2.17±0.22 and 2.29±0.32 units/mg of protein, respectively, whereas the activity in the untreated normal rats was 5.19±0.20 units/mg of protein ($^{+}P<0.05$). However, oral administration of astaxanthin (1, 5, and 25 mg/kg B.W.) for 2 weeks increased superoxide dismutase activity in a dose-dependent manner. Specifically, treatment with 5 and 25 mg of astaxanthin/kg B.W. for 2 weeks significantly ($^{*}P<0.05$, $^{**}P<0.01$) increased superoxide dismutase activity, compared to that in the control rats (Fig. 3).

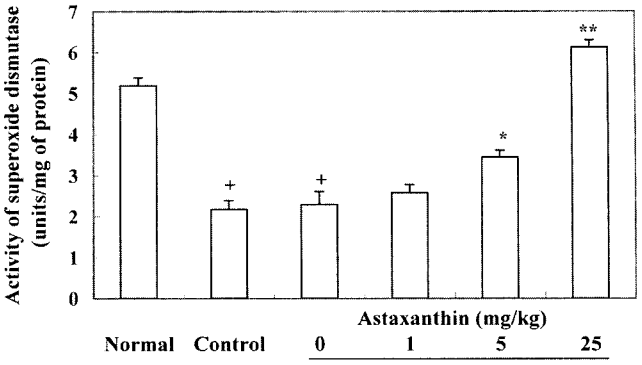

Fig. 3. Effect of astaxanthin on superoxide dismutase activity in naproxen-induced gastric antral ulceration.
The rats were treated with a vehicle (astaxanthin, 0 mg/kg) and three doses of astaxanthin (1, 5, and 25 mg/kg B.W., respectively) once daily for 2 weeks after pretreatment with 80 mg of naproxen/kg B.W. twice daily for 3 days, while the control rats received only 80 mg of naproxen/kg B.W. twice daily for 3 days. Values are expressed as means±S.E.M. of 8 rats. $^{+}P<0.05$, significantly different from the untreated normal rats. $^{*}P<0.05$ and $^{**}P<0.01$, significantly different from the control rats.

The activity of catalase in the control and the vehicle-treated rats was also reduced to 3.44±0.32 and 3.32±0.29 units/mg of protein, respectively, whereas the activity in the untreated normal rats was 5.15±0.25 units/mg of protein ($^{+}P<0.05$). Treatment with 5 and 25 mg of astaxanthin/kg B.W. for 2 weeks significantly ($^{*}P<0.05$, $^{**}P<0.01$) increased catalase activity, compared to that in the control rats (Fig. 4).

The activity of glutathione peroxidase in the control and the vehicle-treated rats was reduced to 5.69±0.60 and 6.04±0.83 μmol/min/mg of protein, respectively, whereas the activity in the untreated normal rats was 13.51±0.81 μmol/min/mg of protein ($^{+}P<0.05$). Treatment with 5 and 25 mg

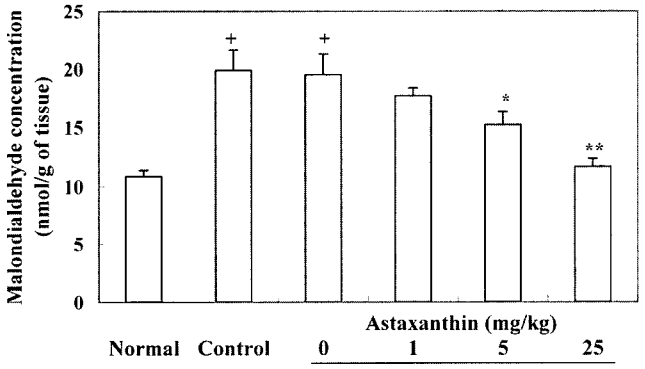

Fig. 2. Effect of astaxanthin on malondialdehyde concentration in naproxen-induced gastric antral ulceration.
The rats were treated with a vehicle (astaxanthin, 0 mg/kg) and three doses of astaxanthin (1, 5, and 25 mg/kg B.W., respectively) once daily for 2 weeks after pretreatment with 80 mg of naproxen/kg B.W. twice daily for 3 days, while the control rats received only 80 mg of naproxen/kg B.W. twice daily for 3 days. Values are expressed as means±S.E.M. of 8 rats. $^{+}P<0.05$, significantly different from the untreated normal rats. $^{*}P<0.05$ and $^{**}P<0.01$, significantly different from the control rats.

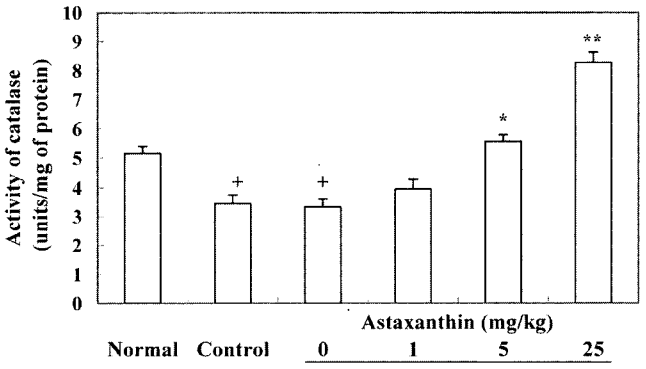

Fig. 4. Effect of astaxanthin on catalase activity in naproxen-induced gastric antral ulceration.
The rats were treated with a vehicle (astaxanthin, 0 mg/kg) and three doses of astaxanthin (1, 5, and 25 mg/kg B.W., respectively) once daily for 2 weeks after pretreatment with 80 mg of naproxen/kg B.W. twice daily for 3 days, while the control rats received only 80 mg of naproxen/kg B.W. twice daily for 3 days. Values are expressed as means±S.E.M. of 8 rats. $^{+}P<0.05$, significantly different from the untreated normal rats. $^{*}P<0.05$ and $^{**}P<0.01$, significantly different from the control rats.

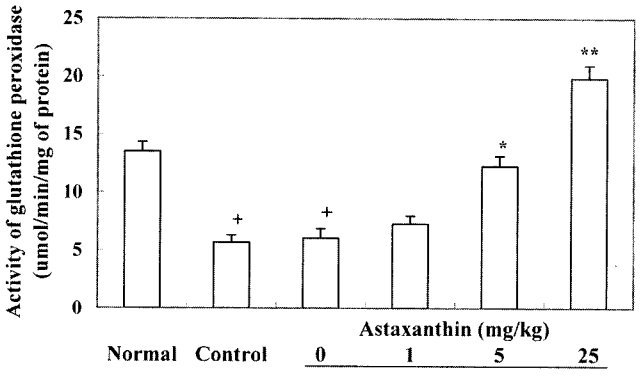

Fig. 5. Effect of astaxanthin on glutathione peroxidase activity in naproxen-induced gastric antral ulceration.
The rats were treated with a vehicle (astaxanthin, 0 mg/kg) and three doses of astaxanthin (1, 5, and 25 mg/kg B.W., respectively) once daily for 2 weeks after pretreatment with 80 mg of naproxen/kg B.W. twice daily for 3 days, while the control rats received only 80 mg of naproxen/kg B.W. twice daily for 3 days. Values are expressed as means±S.E.M. of 8 rats. $^{+}P<0.05$, significantly different from the untreated normal rats. $^{*}P<0.05$ and $^{**}P<0.01$, significantly different from the control rats.

of astaxanthin/kg B.W. for 2 weeks significantly ($^{*}P<0.05$, $^{**}P<0.01$) increased glutathione peroxidase activity, compared to that in the control rats (Fig. 5).

These results indicate that treatment of astaxanthin healed naproxen-induced gastric antral ulcer and removed naproxen-induced lipid peroxides. Astaxanthin also activated superoxide dismutase, catalase, and glutathione peroxidase in a dose-dependent manner.

Discussion

Most NSAIDs, including naproxen, induce severe gastric mucosal lesions in rats [4, 8, 9, 19, 35, 39]. These compounds comprise polar lipids that have a high affinity for the lipophilic areas of cell membranes, where their polar groups trigger membrane disruption, accompanied 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-soluble agents of injury, such as acid, pepsin, and bile salts, which cause lipid peroxidation and also alter membrane fluidity [24]. In this study, naproxen was chosen as the ulceration-causing NSAID in rats, because it is used more frequently than other NSAIDs for arthritic patients, and also because the naproxen-induced gastric antral ulcer model best represents the human situation, where NSAID-induced gastric ulceration occurs mainly in the gastric antrum [23, 24, 32]. The present study investigated the *in vivo* therapeutic effect of astaxanthin, isolated from *Xanthophyllomyces dendrorhous* mutant, on naproxen-induced gastric antral ulceration in rats. Peroxidation of lipids and changes in the activities of healing-related enzymes such as superoxide dismutase, catalase, and glutathione peroxidase were also monitored.

The administration of astaxanthin showed a curative effect on naproxen-induced gastric antral ulcer. An 80 mg of naproxen/kg B.W. for 3 days increased the lipid peroxide level dramatically, and this increase was reduced by treatment with astaxanthin (1, 5, and 25 mg/kg B.W.) for 2 weeks in a dose-dependent manner. Among the three doses of astaxanthin tested, the highest dose (25 mg/kg B.W.) showed the best effect in reducing the lipid peroxide level.

The enzymes such as superoxide dismutase, catalase, and glutathione peroxidase provide defense against the oxidative tissue damage of gastric mucosa after administration of naproxen [31]. The activities of these enzymes were significantly inhibited by naproxen administration, indicating that the inhibition of these enzymatic activities was, at least in part, responsible for the oxidative tissue damage of gastric mucosa after administration of naproxen. On the other hand, the oral administration of astaxanthin for 2 weeks significantly increased the activities of these enzymes in a dose-dependent manner. Specifically, the highest dose (25 mg/kg B.W.) of astaxanthin showed drastic increase of superoxide dismutase, catalase, and glutathione peroxidase activities up to the normal level (untreated normal rats). These results clearly revealed that astaxanthin heals the rat gastric mucosa by its ability to increase the activities of free radical scavenging enzymes such as superoxide dismutase, catalase, and glutathione peroxidase in the mucosa. Macroscopically, 25 mg of astaxanthin/kg B.W. also reduced the depth and severity of the naproxen-induced gastric antral ulcer.

In conclusion, astaxanthin showed a therapeutic effect on naproxen-induced gastric antral ulcer in a dose-dependent manner. Thus, our results led us to conclude that astaxanthin is one of the powerful remedies of gastric antral ulcer through inhibition of lipid peroxidation and activation of superoxide dismutase, catalase, and glutathione peroxidase. We suggest that use of astaxanthin may offer an attractive new treatment strategy for curing gastric lesions in humans.

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