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Protective effect of astaxanthin on naproxen-induced gastric antral ulceration in rats

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Abstract

Frequently used for humans as non-steroidal anti-inflammatory drug, naproxen has been known to induce ulcerative gastric lesion. The present study investigated the in vivo protective effect of astaxanthin isolated from *Xanthophyllomyces dendrorhous* against naproxen-induced gastric antral ulceration in rats. The oral administration of astaxanthin (1, 5, and 25 mg/kg of body weight) showed a significant protection against naproxen (80 mg/kg of body weight)-induced gastric antral ulcer and inhibited elevation of the lipid peroxide level in gastric mucosa. In addition, pretreatment of astaxanthin resulted in a significant increase in the activities of radical scavenging enzymes such as superoxide dismutase, catalase, and glutathione peroxidase. A histologic examination clearly proved that the acute gastric mucosal lesion induced by naproxen nearly disappeared after the pretreatment of astaxanthin. These results suggest that astaxanthin removes the lipid peroxides and free radicals induced by naproxen, and it may offer potential remedy of gastric ulceration.

Keywords: Anti-ulcer drug; Astaxanthin; Gastric antral ulceration; Orogastric administration; *Xanthophyllomyces dendrorhous*

1. Introduction

Many NSAIDs (non-steroidal anti-inflammatory drugs) have been widely used clinically as anti-inflammatory, analgesic agents. However, ulcerative lesions of the gastrointestinal tract are one of the major side effects of NSAIDs, and they are the major limitation to their use as anti-inflammatory drugs (Lanza, 1984; Beck et al., 1990; Bjarnason et al., 1993; Tenenbaum, 1999). In the present 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 is suitable in the human situation, where NSAID-induced gastric ulceration occurs mainly in gastric antrum (Cioli et al., 1979; Lanza, 1984; Roth and Bennett, 1987; Suwa et al., 1987; Beck et al., 1990; McCarthy, 1990, 1995; Calhoun et al., 1992; Tenenbaum, 1999). 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 an adverse effect (Suwa et al., 1987; Calhoun et al., 1992). Production of oxygen free radicals and lipid peroxidation play a crucial role in the development of the gastric antral ulceration induced by naproxen (Parks, 1989; Yoshikawa et al., 1990).

Astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) is widely distributed in nature, and it 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* (Andrew et al.,

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1976; Johnson and Lewis, 1979). *X. dendrorhous* is a carotenoid-producing yeast, which synthesizes astaxanthin as its main carotenoid (Johnson and Lewis, 1979). However, *X. dendrorhous* wild-type is uneconomical because of its low astaxanthin content and high production cost. Therefore, astaxanthin-overproducing *X. dendrorhous* mutant developed in our laboratory was used (Kim et al., 2004).

Astaxanthin has important metabolic functions in animals, including conversion to vitamin A (Bendich and Olson, 1989), enhancement of immune response (Jyonouchi et al., 1994; Okai and Higashi-Okai, 1996), and protection against diseases such as cancer by scavenging oxygen radicals (Tanaka et al., 1994, 1995a,b; Jyonouchi et al., 2000). 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 (Krinsky, 1989; Kurashige et al., 1990; Naguib, 2000; Mortensen et al., 2001). These effects are considered to be defense mechanisms against the attack of reactive oxygen species. Astaxanthin also shows a strong activity as inhibitor of oxygen radical-mediated lipid peroxidation (Lim et al., 1992; Palozza and Krinsky, 1992).

The aim of this study is to determine the protective effect of astaxanthin on naproxen-induced gastric antral ulceration in rats, and to investigate the mechanisms of astaxanthin for possible gastroprotection by measuring the amount of lipid peroxidation and by comparing the activities of enzymatic scavengers such as superoxide dismutase, catalase, and glutathione peroxidase.

2. Materials and methods

2.1. Yeast strains and astaxanthin extraction

X. dendrorhous ATCC 96594 was provided by Korea Research Institute of Bioscience and Biotechnology. The JH-1, astaxanthin-overproducing mutant, was derived from *X. dendrorhous* ATCC 96594 by mutagenesis with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG) (An et al., 1989; Lewis et al., 1990; Kim et al., 2004). For routine analysis of astaxanthin, the washed cell pellets were mixed with dimethyl sulfoxide preheated 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 high performance liquid chromatography (HPLC) (Sedmak et al., 1990). The results of HPLC indicated that the purity of astaxanthin was more than 95% (Kim et al., 2004).

2.2. Animals

Male Sprague–Dawley rats (230~250 g, 7 weeks old) were purchased from Daehan Biolink Co., Ltd. Rats were placed singled 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 they 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.

2.3. Chemicals

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

2.4. Induction and evaluation of naproxen-induced gastric ulceration

Naproxen (40–100 mg/kg dose range) was administered to rats by orogastric gavage twice daily (at 07.00 h and 17.00 h). Rats were killed under deep ether anesthesia at various time points (1–5 days) after naproxen administration. The stomach was opened along the greater curvature, and then rinsed in 0.9% saline. Gastric antral ulcers were counted with the aid of a 7× magnifier and an attached metric scale. The area (in mm^2) of individual gastric ulcers was also estimated by determining the product of the measured ulcer length and its width. Gastric antral ulceration was confirmed by routine histological evaluation of the samples from several rat stomachs. These initial studies were solely designed to help determine an optimal gastric damaging dose as well as time point for subsequent anti-ulcer drug testing against naproxen-induced gastric antral damage.

2.5. Evaluation of astaxanthin effect, as anti-ulcer drug

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/kg of naproxen twice daily (at 07.00 h and 17.00 h) for 3 days. Each of the remaining four groups was treated with 0, 1, 5, and 25 mg/kg body weight of astaxanthin twice daily (at 07.00 h and 17.00 h) for 3 days, and then treated with 80 mg/kg of naproxen twice daily (at 07.00 h and 17.00 h) for 3 days. All the rats were killed under deep ether anesthesia

4 h after the naproxen treatment. 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 (1955). The quantitative analysis of protein was measured by Bradford protein assay.

2.6. 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. (1979). 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 supplemented with *n*-butanol and pyridine (15:1 v/v), shaken vigorously for 1 min, and centrifuged for 10 min at $3500 \times g$. 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 malondialdehyde per g of weight tissue.

2.7. 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 (1967). 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 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.

2.8. Measurement of catalase activity

The activity of catalase in gastric mucosa of rats was determined according to the method of Aebi (1974). 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 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.

2.9. 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 (1976). The reaction mixture consisted of glutathione peroxidase assay buffer (50 mM potassium phosphate buffer pH 8.0, 0.5 mM EDTA) and NADPH assay reagent (5 mM NADPH, 42 mM reduced glutathione, 10 units/ml glutathione reductase). A sample of supernatant fluid with homogenate solution and 50 mM potassium phosphate buffer at pH 7.5 was prepared by centrifuging it at $1000 \times g$ for 10 min at 4 °C. The cuvette was subsequently added 900 μl of glutathione peroxidase assay buffer, 50 μl of NADPH assay reagent, and 50 μl of sample, and mixed by inversion. The reaction started when 10 μl of 30 mM *tert*-butyl hydroperoxide or 80% cumene hydroperoxide was added. Absorbance was recorded by the following program; Wavelength: 340 nm/Initial delay: 15 s/Interval: 10 s/Number of readings: 6. The activity of enzyme was the sum of data obtained using 30 mM *tert*-butyl hydroperoxide and 80% cumene hydroperoxide. The level of glutathione was expressed in terms of μmol/min/mg of protein.

2.10. 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 standard procedures.

2.11. 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).

3. Results

3.1. Determination of optimal naproxen-induced gastric antral ulcer model in rats

Gastric damage was found to be primarily in the form of antral ulcers. Ulceration was judged macroscopically by clear depth of penetration into the gastric mucosal surface. The mean antral ulcer area in all naproxen (40–100 mg/kg dose range)-treated rats was subsequently increased for 3 days of naproxen administration (Fig. 1). However, a relatively high mortality rate of 25% was observed in rats

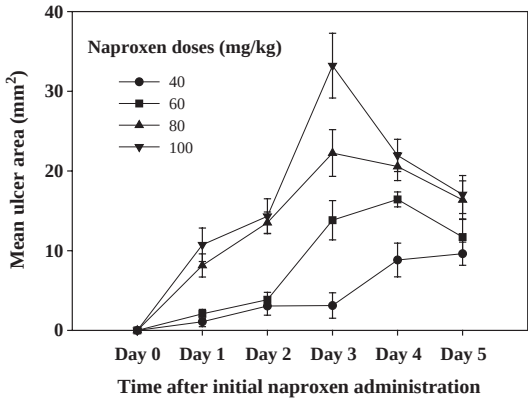

Fig. 1. Ulcerogenic effects in rats with various doses of naproxen, when administered over a 5 day period. Naproxen (40–100 mg/kg) was given by orogastric gavage at 07.00 h and 17.00 h. Rats were euthanized at various time points (i.e. up to 5 days) after naproxen dosing. The mean area of gastric ulceration (mm^2) was then determined. $n=3-8$ per treatment group/time point.

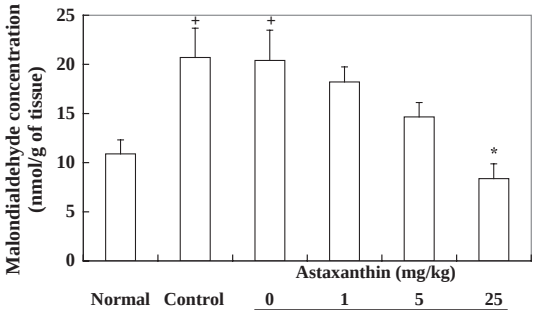

Fig. 3. Effect of astaxanthin on malondialdehyde concentration in naproxen-induced gastric antral ulceration. The rats were treated with 0, 1, 5, and 25 mg/kg body weight of astaxanthin twice daily (at 07.00 h and 17.00 h) for 3 days, and then treated with 80 mg/kg of naproxen twice daily (at 07.00 h and 17.00 h) for 3 days. The control rats received only 80 mg/kg of naproxen for 3 days. Values are expressed as means±S.E.M. of 8 rats. $^{+}P<0.05$, significantly different from the normal rats. $^{*}P<0.05$, different from the control rats.

treated with a 100 mg/kg dose of naproxen. In contrast, a low mortality rate of 5% was observed in rats treated with an 80 mg/kg dose. A gastric antral ulcer was also clearly visible in rats receiving this dose of naproxen (Fig. 2). Therefore, an 80 mg/kg dose of naproxen for 3 days was determined to be optimal for anti-ulcer drug studies. The mean antral ulcer area tended to decrease in all naproxen-treated rats by day 5 of naproxen administration (Fig. 1). These results suggest that an adaptation took place in the rat gastric antrum to repeated naproxen dosing.

3.2. Effect of astaxanthin on naproxen-induced gastric antral ulceration in rats

Superficial or deep erosions, bleeding, and antral ulcers were observed in rats receiving the 80 mg/kg dose of naproxen for 3 days. However, pretreatment with 25 mg/kg of astaxanthin for 3 days reduced the depth and severity of naproxen-induced gastric antral ulcer (Fig. 2).

The concentration of malondialdehyde in the control (naproxen, 80 mg/kg) and the vehicle (astaxanthin, 0 mg/kg)-pretreated rats was increased to 20.71±2.97 and 20.40±3.08 nmol/g of tissue, respectively, whereas the concentration of malondialdehyde in the untreated normal rats remained at 10.89±1.43 nmol/g of tissue ($^{+}P<0.05$). This increase in the concentration of malondialdehyde was reduced in a dose-dependent manner in all astaxanthin-pretreated rats. Especially, pretreatment with 25 mg/kg of astaxanthin for 3 days showed a significant ($^{*}P<0.05$) decrease in the concentration of malondialdehyde compared to that in the control rats (Fig. 3).

The activity of superoxide dismutase in the control and the vehicle-pretreated rats was reduced to 2.13±0.54 and 2.08±0.45 units/mg of protein, respectively, whereas the activity of superoxide dismutase in the untreated normal rats was 5.27±0.58 units/mg of protein ($^{+}P<0.05$). However, oral administration of astaxanthin (1, 5, and 25 mg/kg of body weight) for 3 days increased superoxide dismutase activity in a dose-dependent manner. Especially, pretreatment with 5, and 25 mg/kg of astaxanthin for 3 days significantly ($^{*}P<0.05$, $^{**}P<0.01$) increased superoxide dismutase activity compared to that in the control rats (Fig. 4).

The activity of catalase in the control and the vehicle-pretreated rats was also reduced to 2.41±0.57 and 2.34±0.60 units/mg of protein, respectively, whereas the activity of catalase in the untreated normal rats was 5.49±0.52 units/mg of protein ($^{+}P<0.05$). Pretreatment with 5, and 25 mg/kg of astaxanthin for 3 days significantly

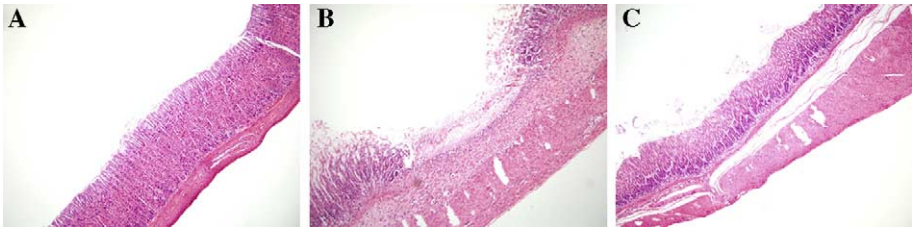

Fig. 2. Protective effect of astaxanthin on naproxen-induced gastric antral ulceration in rats. The rats were treated with 25 mg/kg dose of astaxanthin twice daily for 3 days, and then treated with 80 mg/kg of naproxen twice daily for 3 days. (A) Normal gastric antrum from the untreated normal rat. (B) Gastric antral ulcer in the naproxen-treated rat. A gastric ulcer is clearly visible in the gastric antrum. (C) Gastric antrum in the astaxanthin-pretreated rat. Pretreatment with 25 mg/kg of astaxanthin for 3 days reduced inflammation and gastric antral ulcer.

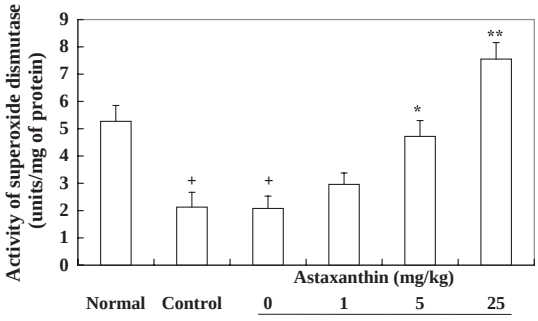

Fig. 4. Effect of astaxanthin on superoxide dismutase activity in naproxen-induced gastric antral ulceration. The rats were treated with 0, 1, 5, and 25 mg/kg body weight of astaxanthin twice daily (at 07.00 h and 17.00 h) for 3 days, and then treated with 80 mg/kg of naproxen twice daily (at 07.00 h and 17.00 h) for 3 days. The control rats received only 80 mg/kg of naproxen for 3 days. Values are expressed as means±S.E.M. of 8 rats. $^{+}P<0.05$, significantly different from the normal rats. $^{*}P<0.05$ and $^{**}P<0.01$, significantly different from the control rats.

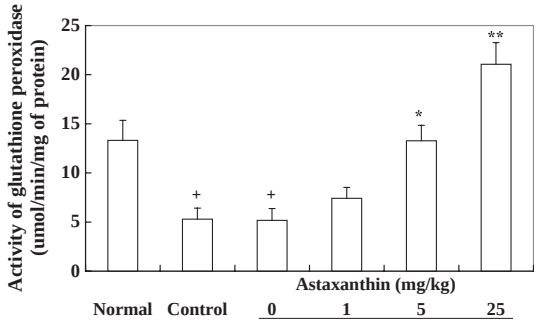

Fig. 6. Effect of astaxanthin on glutathione peroxidase activity in naproxen-induced gastric antral ulceration. The rats were treated with 0, 1, 5, and 25 mg/kg body weight of astaxanthin twice daily (at 07.00 h and 17.00 h) for 3 days, and then treated with 80 mg/kg of naproxen twice daily (at 07.00 h and 17.00 h) for 3 days. The control rats received only 80 mg/kg of naproxen for 3 days. Values are expressed as means±S.E.M. of 8 rats. $^{+}P<0.05$, significantly different from the normal rats. $^{*}P<0.05$ and $^{**}P<0.01$, significantly different from the control rats.

(*$P<0.05$, **$P<0.01$) increased catalase activity compared to that in the control rats (Fig. 5).

The activity of glutathione peroxidase in the control and the vehicle-pretreated rats was reduced to 5.29±1.13 and 5.17±1.20 μmol/min/mg of protein, respectively, whereas the activity of glutathione peroxidase in the untreated normal rats was 13.31±2.04 μmol/min/mg of protein ($^{+}P<0.05$). Pretreatment with 5, and 25 mg/kg of astaxanthin for 3 days significantly (*$P<0.05$, **$P<0.01$) increased glutathione peroxidase activity compared to that in the control rats (Fig. 6).

These results indicated that administration of astaxanthin reduced naproxen-induced gastric antral ulcer and removed the lipid peroxides induced by naproxen. Astaxanthin also activated superoxide dismutase, catalase, and glutathione peroxidase in a dose-dependent manner. Pretreatment with 25 mg/kg of astaxanthin for 3 days completely protected the gastric mucosa against the loss of the enzyme, resulting in a significant increase of the enzymatic superoxide dismutase, catalase, and glutathione peroxidase activities to the level of the untreated normal rats.

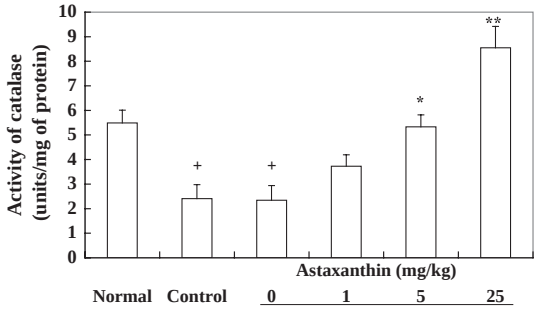

Fig. 5. Effect of astaxanthin on catalase activity in naproxen-induced gastric antral ulceration. The rats were treated with 0, 1, 5, and 25 mg/kg body weight of astaxanthin twice daily (at 07.00 h and 17.00 h) for 3 days, and then treated with 80 mg/kg of naproxen twice daily (at 07.00 h and 17.00 h) for 3 days. The control rats received only 80 mg/kg of naproxen for 3 days. Values are expressed as means±S.E.M. of 8 rats. $^{+}P<0.05$, significantly different from the normal rats. $^{*}P<0.05$ and $^{**}P<0.01$, significantly different from the control rats.

4. Discussion

Various NSAIDs induce severe gastric mucosal damage in rats (Cioli et al., 1979; Lanza, 1984; Suwa et al., 1987; Beck et al., 1990; Calhoun et al., 1992; Tenenbaum, 1999). These compounds comprise 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. However, most NSAID-induced gastric damage occurs mainly in the corpus region of the stomach and tends to be mostly in the form of erosions rather than ulcers. This is unlike the situation in humans, where NSAID-induced gastric ulceration occurs mainly in gastric antrum (Roth and Bennett, 1987; McCarthy, 1990, 1995). In the present study, we used a simple, reproducible and relevant naproxen-induced gastric antral ulcer model, which is suitable for the human situation. The gastric antral ulcer area was increased in all naproxen (40–100 mg/kg dose range)-treated rats for the first 3 days of naproxen administration, and an 80 mg/kg dose of naproxen for 3 days was found to be optimal for anti-ulcer drug studies. This dose of naproxen for 3 days clearly showed gastric antral ulcer and a low mortality rate in rats. The result was used in the evaluation of astaxanthin effect against naproxen-induced gastric antral ulcer.

The administration of astaxanthin showed a significant protection against naproxen-induced gastric antral ulcer. An 80 mg/kg dose of naproxen for 3 days increased the lipid peroxide level dramatically, and this increase was prevented by pretreatment of astaxanthin (1, 5, and 25 mg/kg of body weight) for 3 days in a dose-dependent manner. Among the three doses of astaxanthin tested, the highest dose (25 mg/kg of body weight) 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 (Parks, 1989). The activities of these enzymes were significantly inhibited by naproxen administration, which indicated that 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 3 days significantly increased the activities of these enzymes in a dose-dependent manner. Especially, the highest dose (25 mg/kg of body weight) of astaxanthin completely protected the gastric mucosa against the loss in the enzyme, resulting in a drastic increase of the enzymatic superoxide dismutase, catalase, and glutathione peroxidase activities up to the normal level (untreated normal rats). These results clearly revealed that astaxanthin protects 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, a 25 mg/kg dose of astaxanthin also reduced the depth and severity of the naproxen-induced gastric antral ulcer.

In conclusion, the administration of naproxen (80 mg/kg of body weight) to rats proved to be a reliable and relevant method for evaluating NSAID-induced gastric antral ulceration. Astaxanthin showed a protective effect on naproxen-induced gastric antral ulcer in a dose-dependent manner. Thus, our results suggest that astaxanthin is one of the powerful remedies of gastric antral ulcer, inhibiting lipid peroxidation, and that it activates 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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