Suppressive Effect of Astaxanthin Isolated from the *Xanthophyllomyces dendrorhous* Mutant on Ethanol-Induced Gastric Mucosal Injury in Rats

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Ethanol has been found to induce ulcerative gastric lesion in humans. The present study investigated the in vivo protective effect of astaxanthin isolated from the *Xanthophyllomyces dendrorhous* mutant against ethanol-induced gastric mucosal injury in rats. The rats were treated with 80% ethanol for 3 d after pretreatment with two doses of astaxanthin (5 and 25 mg/kg of body weight respectively) for 3 d, while the control rats received only 80% ethanol for 3 d. The oral administration of astaxanthin (5 and 25 mg/kg of body weight) showed significant protection against ethanol-induced gastric lesion and inhibited elevation of the lipid peroxide level in gastric mucosa. In addition, pretreatment with 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 indicated that the acute gastric mucosal lesion induced by ethanol nearly disappeared after pretreatment with astaxanthin.

Key words: anti-ulcer drug; astaxanthin; gastric mucosal injury; orogastric administration; *Xanthophyllomyces dendrorhous*

Ethanol is known to produce erosions, ulcerative lesions, and petechial bleeding in the mucosa of the stomach in humans.1-3) Oral administration of ethanol to rats rapidly induces gastric mucosal lesions, which are commonly used to study both the pathogenesis of and therapy for human ulcerative disease. Ethanol rapidly penetrates the gastric mucosa, and it causes membrane damage, exfoliation of cells, erosion, and ulcer formation.4) It has been suggested that ethanol-induced gastric damage is mediated by the generation of free radicals.5-8) Recently, astaxanthin (3,3’-dihydroxy-β,β’-carotene-4,4’-dione) has been documented to provide important metabolic functions in animals, including conversion to vitamin A,9) enhancement of immune response,10,11) and protection against diseases such as cancer by scavenging oxygen radicals.12-16) 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.17-20) These effects are considered to be defense mechanisms against attack by reactive oxygen species. Astaxanthin also shows strong activity as an inhibitor of oxygen radical-mediated lipid peroxidation.21,22)

The aim of this study was to determine the protective effect of astaxanthin isolated from the *Xanthophyllomyces dendrorhous* mutant against ethanol-induced gastric mucosal injury 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.

Materials and Methods

*Yeast strains and astaxanthin extraction.* X. *dendrorhous* ATCC 96594 was provided by the Korea Research Institute of Bioscience and Biotechnology. The astaxanthin-overproducing mutant JH1 was derived from *X. dendrorhous* ATCC 96594 by mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine.23) For routine analysis of astaxanthin, the washed cell pellets were mixed with dimethyl sulfoxide preheated to 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 the concentrate and quantitatively analyzed by HPLC.23,24) The results of HPLC indicated that the astaxanthin was purified (Fig. 1).

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Animals. Male Sprague-Dawley rats (230–250 g, 7 weeks old) were purchased from Daehan Biolink, Seoul, Korea. Rats were placed in cages with wire-net floors in a controlled room (temperature 22–24 °C, humidity 70–75%, light on at 06.00 h and off at 18.00 h; 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 ethanol, rats were provided food for the remainder of the study. Rats were also allowed tap water throughout the study period. The animal experiments were conducted in accordance with guidelines established by the Animal Care and Use Committee of Korea University, and were approved by the committee.

Chemicals. All chemicals were of the highest purity available. Absolute ethanol was obtained from Sigma Chemical (St. Louis, MO). Ethanol was administered by orogastric gavage, with an appropriate feeding needle at a volume of 5 ml/kg. Astaxanthin was dissolved in medium chain triglyceride solution (the vehicle) immediately before use and administered intragastrically to rats at a volume of 5 ml/kg.

Evaluation of astaxanthin effect as anti-ulcer drug. To evaluate the effect of astaxanthin, the rats were divided into five groups (n = 8 rats per group). The untreated normal rats received distilled water for 3 d, in comparable volume by the oral route. The control rats received 80% ethanol only for 3 d. Each of the remaining three groups was treated with 80% ethanol for 3 d after pretreatment with 0, 5, or 25 mg/kg body weight of astaxanthin for 3 d. All the rats were killed under deep ether anesthesia 1 h after the last oral administration of ethanol. The 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. The quantitative analysis of protein was measured by Bradford protein assay.

Measurement of lipid peroxidation. Lipid peroxidation was determined by measuring the content of malondialdehyde in the gastric mucosa according to a modification of the method of Ohkawa et al. The stomach homogenate was supplemented with 8.1% sodium dodecyl sulfate, 20% acetic acid (pH 3.5), and 0.8% thiobarbituric acid, 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 g. Absorbance was measured at 532 nm. Lipid peroxidation was calculated from the standard curve using the malondialdehyde tetrabutylammonium salt and expressed as the content of nmol malondialdehyde per g of weight tissue.

Measurement of superoxide dismutase activity. The activity of superoxide dismutase in the gastric mucosa of rats was determined according to the method of McCord and Fridovich. 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.

Measurement of catalase activity. The activity of catalase in the gastric mucosa of rats was determined according to the method of Aebi. The standard assay was performed in 3 ml of 50 mM potassium phosphate buffer at pH 7.0 (1.9 ml) containing 10 mM H₂O₂ (1 ml) and tissue homogenate (100 µl). Under these conditions, the amount of catalase required to decompose 1.0 µmol of H₂O₂ 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.

Measurement of glutathione peroxidase activity. The activity of glutathione peroxidase in the gastric mucosa of rats was determined by a modification of the method of Lawrence and Burk.\textsuperscript{30} 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 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 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 standard procedures.

Statistical analysis. All values were represented as means ± S.E.M. Data were analyzed by ANOVA according to the 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\textsuperscript{®} software package (Release 8.02, 2001).

Results

Effect of astaxanthin on ethanol-induced gastric mucosal injury in rats

Gastric lesions were judged macroscopically by clear depth of penetration into the gastric mucosal surface. Superficial or deep erosions, bleeding, and ulcers were observed in rats receiving 80% ethanol for 3 d. But, pretreatment with 25 mg/kg of astaxanthin for 3 d reduced the depth and severity of ethanol-induced gastric mucosal lesions. Mild to moderate villous atrophy was noted, but no severe necrotic changes in mucus ridges were observed (Fig. 2, Fig. 3).

The content of malondialdehyde in the control (ethanol, 80%) and the vehicle-pretreated (astaxanthin, 0 mg/kg) rats increased to 19.94 ± 2.15 and 19.96 ± 1.97 nmol/g of tissue respectively, whereas the content of malondialdehyde in the untreated normal rats remained at 10.36 ± 0.76 nmol/g of tissue ($^\ast P < 0.05$). This increase in the content of malondialdehyde declined in a dose-dependent manner in all astaxanthin-pretreated rats. Especially, pretreatment with 25 mg/kg of astaxanthin for 3 d showed a significant ($^\ast P < 0.05$) decrease in the content of malondialdehyde compared to that in the control rats (Fig. 4).

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The activity of superoxide dismutase in the control and the vehicle-pretreated rats declined to 2.51 ± 0.47 and 2.14 ± 0.34 units/mg of protein respectively, whereas the activity of superoxide dismutase in the untreated normal rats was 5.87 ± 0.48 units/mg of protein (*P < 0.05), but pretreatment with 5 and 25 mg/kg of astaxanthin for 3 d dose-dependently increased superoxide dismutase activity compared to that in the control rats. The effect was significant (*P < 0.05) at 25 mg/kg of astaxanthin (Fig. 5).

The activity of catalase in the control and the vehicle-pretreated rats also declined to 2.89 ± 0.56 and 2.77 ± 0.43 units/mg of protein respectively, whereas the activity of catalase in the untreated normal rats was 5.12 ± 0.45 units/mg of protein (*P < 0.05). Pretreatment with 5 and 25 mg/kg of astaxanthin for 3 d significantly (*P < 0.05, **P < 0.01) increased catalase activity compared to that in the control rats (Fig. 6).

The activity of glutathione peroxidase in the control and the vehicle-pretreated rats declined to 5.71 ± 1.11 and 5.28 ± 1.26 μmol/min/mg of protein respectively, whereas the activity of glutathione peroxidase in the untreated normal rats was 13.91 ± 1.50 μmol/min/mg of protein (*P < 0.05). Pretreatment with 5 and 25 mg/kg of astaxanthin for 3 d significantly (*P < 0.05, **P < 0.01) increased glutathione peroxidase activity compared to that in the control rats (Fig. 7).
These results suggest that pretreatment with astaxanthin prevented ethanol-induced mucosal changes and removed ethanol-induced lipid peroxides. Astaxanthin also activated superoxide dismutase, catalase, and glutathione peroxidase in a dose-dependent manner (Fig. 8).

Discussion

Ethanol induces severe gastric mucosal damage in humans and rodents. It rapidly penetrates the gastric mucosa, causing membrane damage, exfoliation of cells, and erosions. The subsequent increase in mucosal permeability together with the release of vasoactive products from mast cells, macrophages, and other blood cells can lead to vascular injury, necrosis, and ulcer formation. It has been suggested that production of oxygen free radicals plays a crucial role in the development of ethanol-induced gastric lesions. The present study investigated the in vivo protective effect of astaxanthin isolated from the Xanthophyllomyces dendrorhous mutant against ethanol-induced gastric mucosal injury 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.

Administration of astaxanthin showed significant protection against ethanol-induced gastric mucosal injury. Treatment with 80% ethanol for 3 d increased the lipid peroxide level dramatically, and this increase was prevented by pretreatment with astaxanthin (5 and 25 mg/kg of body weight) for 3 d in a dose-dependent manner. Especially, pretreatment with 25 mg/kg of astaxanthin for 3 d showed the best effect in reducing the lipid peroxide level.

Enzymes such as superoxide dismutase, catalase, and glutathione peroxidase provide defense against oxidative tissue damage to gastric mucosa after administration of ethanol. On the other hand, oral administration of astaxanthin for 3 d significantly increased the activities of these enzymes in a dose-dependent manner. Especially, the higher dose (25 mg/kg of body weight) of astaxanthin showed drastic increases in superoxide dismutase, catalase, and glutathione peroxidase activities compared to those in the untreated normal rats. These results clearly indicate 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 (Fig. 8). Macroscopically, the 25 mg/kg dose of astaxanthin also reduced the depth and severity of ethanol-induced gastric mucosal lesions.

It has been reported that astaxanthin inhibits urinary bladder carcinogenesis induced by N-butyl-N-(4-hydroxybutyl) nitrosamine in mice, oral carcinogenesis induced by 4-nitrosoguanosine 1-oxide in rats, and colon carcinogenesis induced by azoxymethane in rats. These results suggest that the protective effect of astaxanthin is related to its effectiveness as an antioxidant and free-radical scavenger.

In conclusion, astaxanthin showed a protective effect on ethanol-induced gastric mucosal injury in a dose-dependent manner. These results lead us to believe that astaxanthin is a powerful remedy for gastric mucosal lesions, inhibiting lipid peroxidation, and that it activates superoxide dismutase, catalase, and glutathione peroxidase. We suggest that use of astaxanthin might offer an attractive new treatment strategy for curing gastric lesions in humans.

References

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