Preventive Effects of Laminaria japonica Aqueous Extract on the Oxidative Stress and Xanthine Oxidase Activity in Streptozotocin-Induced Diabetic Rat Liver

Da-Qing Jin, Gao Li, Jin-Sook Kim, Chul-Soon Yong, Jung-Ae Kim,* and Keun Huh

College of Pharmacy, Yeungnam University; 214–1 Dae-dong, Gyeongsan 712–749, Korea.

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Increasing evidence in both experimental and clinical studies suggests that oxidative stress plays a major role in the pathogenesis of diabetes mellitus type 1 and type 2. Xanthine oxidase (XO) has been proposed as one of the sources of free radical formation in diabetes. We therefore investigated the preventive effects of Laminaria japonica aqueous extract (LJE) on alterations in the activity of hepatic XO and oxidative stress in the streptozotocin-induced experimental diabetes. We found that lipid peroxide levels and xanthine oxidase activity were increased, whereas glutathione (GSH), GSH reductase and GSH peroxidase were decreased in the liver of streptozotocin-induced diabetic rats. Pretreatment with LJE of 100 mg/kg orally for 5 d significantly reduced blood glucose levels and hepatic lipid peroxidation in the diabetic rats. In addition, the content of glutathione was restored to the control level by LJE pretreatment. Furthermore, LJE significantly suppressed the increased activity of XO and type conversion of the xanthine dehydrogenase to XO in diabetic rat liver. The results suggest that Laminaria japonica would be of great value in preventing hyperglycemia in diabetes mellitus as a dietary supplement possibly, through its antioxidant activity.

Key words Laminaria japonica; diabetes; xanthine oxidase; oxidative damage; streptozotocin

Oxidative stress resulting from the imbalance between free radical generating systems and scavenging systems1,2) has been involved in the pathogenesis of complications in many tissues of diabetes mellitus.3) The increase in superoxide radical and/or hydrogen peroxide has been observed in the blood of diabetic patients, which is believed to play a role in the development of vascular complications.4) The depletion of glutathione (GSH) also occurs in diabetic endothelial cells.5,6) GSH participates in various biological processes such as the metabolism of sulfur-containing amino acids and biosynthesis of DNA, and in the cellular defense system against oxidative stress by scavenging free radicals and reactive oxygen species.7) In addition, hepatic glutathione has been shown to be critical for hepatic insulin-sensitizing substance action.8)

Even though the exact mechanism of the formation of oxidative stress has not been studied, auto-oxidative glycosylation and ketone bodies are proposed as important sources for the free radical generation in type 1 diabetes.5) Recently, it has been reported that xanthine oxidase (XO) shed from the liver is involved in the generation of oxygen free radicals and oxidative stress, resulting in vascular complication of type 1 diabetes,8) the process of which is also well known in pathophysiological conditions of other diseases.9,10) In mammal, XO is synthesized as a dehydrogenase form which uses NAD+ as an electron acceptor. However, xanthine dehydrogenase (XD) is converted into an oxidase form under conditions of tissue injury.

Laminaria japonica thallus has long been used as a Korean folk remedy to promote maternal health. In addition, as a dietary supplement it has been known for several biological activities: hypotensive effect,1,12) scavenging activity against 1,1-diphenyl-1-2-picrylhydrazyl (DPPH) free radicals,13) antimutagenic activity,10) and down-regulation of blood glucose in diabetic rats.14)

In this study, we report the effects of Laminaria japonica aqueous extract (LJE) on oxidative damage induced by streptozotocin in rat liver with regard to the GSH system and XO activity and type conversion of the XD to XO.

MATERIALS AND METHODS

Plant Material Dried thalli of Laminaria japonica were purchased from a folk medicine market “Yak-ryong-si” in Daegu. A voucher specimen (YNP-97-01) is preserved at the College of Pharmacy, Yeungnam University, Gyeongsan, Republic of Korea.

Preparation of L. japonica Aqueous Extract (LJE) Dried thalli of Laminaria japonica (4 kg) were extracted with distilled water held at 100 °C for 4 h, evaporated to dryness (540 g), and kept at 4 °C.

Experimental Animals and Streptozotocin-Induced Diabetes Male Sprague-Dawley rats (Life Science Co., Korea) weighing 200±20 g were housed 5 per cage in a room maintained at 22±2 °C with an alternating 12 h light–dark cycle. Rats had food pellets and tap water ad libitum and were kept in these facilities for at least 2 d before the experiments. They were then divided into four groups of 10 rats each. LJE was dissolved in saline, and given orally by gavage for 5 d at a daily dose of 100 mg/kg. Diabetes in rats was induced by a single intramuscular injection of streptozotocin at a dose of 55 mg/kg body weight in 0.1 M citrate buffer pH 4.5. The control group was treated with saline orally for 5 d and the second group was administered with LJE (100 mg/kg body weight) orally once a day for 5 d. The third diabetic group was injected with streptozotocin (55 mg/kg). The last group was pretreated with LJE (100 mg/kg) orally and 5 d later streptozotocin (55 mg/kg) was injected intramuscularly. Five days after the streptozotocin injection, the animals were sacrificed and blood and liver were collected.

Determination of Blood Glucose Level Hyperglycemic incidence was monitored using glucose oxidase method and expressed in milligrams per deciliter (mg/dl).15)
Determination of Antioxidant Status All groups of rats were sacrificed by decapitation, and the liver was isolated separately and then homogenized with a glass/teflon homogenizer in 4 volumes of 0.1 M potassium phosphate buffer (pH = 7.4). Homogenates were centrifuged (Beckman, U.S.A.) at 15000×g for 1 h and supernatants were used as enzymatic sources in the assay for activities. Glutathione peroxidase activity was assayed by the method of Paglia and Valentine using H2O2 as the substrate. Enzyme activity was expressed as oxidized NADPH nmol/mg protein/min. Glutathione reductase activity was measured by following the oxidation of NADPH at 340 nm. Superoxide dismutase (SOD) activity was measured spectrophotometrically according to the method of Flohe and Otting. In this method, inhibition of the cytochrome c reduction rate is monitored at 550 nm at 25 °C utilizing the xanthine/xanthine oxidase system as a source of superoxide anion. SOD competes for superoxide and decreases the reduction rate of cytochrome c. One unit of SOD activity was defined as the amount of enzyme that inhibits by 50% the rate of cytochrome c reduction. Catalase activity was assayed according to the method of Aebi by measuring the decrease in absorbance of H2O2 at 240 nm for 1 min.

Xanthine Oxidase and Xanthine Dehydrogenase Activities Xanthine oxidase (XO) and xanthine dehydrogenase (XD) activity were measured spectrophotometrically by measuring the amount of uric acid formed from xanthine sodium in the presence of NAD. Ab-XO utilizes NADH as a cofactor in the reaction mixture, which contained 0.1 M potassium phosphate buffer (pH 7.4), 0.1 ml of enzyme source, 0.06 mM of the substrate. In a parallel reaction, xanthine oxidase activity was determined by measuring the rates of uric acid formation in the reaction mixture from xanthine as substrate without NAD+. The reaction was carried out at 37 °C for 15 min. The type conversion ratio from XD to XO was represented as XO/XD+ XO.

Glutathione Level Determination Reduced GSH was measured by the modified method of Griffith. Tissue homogenates were deproteinized by addition of 4% sulfosalicylic acid. Supernant was incubated with 6 nm 5,5'-dithiobis(2-nitrobenzoic acid), 0.3 nm NADPH, 50 units GSH reductase, and 0.1 M sodium phosphate buffer (pH = 7.5). Absorbance of p-nitrothiophenol was measured by a spectrophotometer (Spectronic Genisis 5, Milton Roy, U.S.A.) at 412 nm as an index of the content of GSH. The level of GSH was expressed as µmol of GSH/g tissue.

Lipid Peroxidation Assay Lipid peroxidation was measured by a modified method of Okahwa et al. The tissue homogenate, prepared in a same way as in the section of enzyme assay, was incubated with a reaction buffer containing 8.1% sodium dodecyl sulfate, 20% acetate buffer (pH = 3.5) and 0.8% thiobarbituric acid; the mixture was incubated at 95 °C for 60 min and then cooled at room temperature. Thiobarbituric acid reactive substance in the reactant was transferred into a mixture of n-butanol: pyridine (15:1, v/v). The upper organic layer containing malondialdehyde (MDA) produced by lipid peroxidation was measured by a spectrophotometer at 532 nm. The level of lipid peroxidation was expressed as nmol MDA/mg protein. The protein concentration was measured by the method of Lowry et al. using bovine serum albumin as the standard.

Statistical Analysis Data were expressed as mean±standard deviation of the mean (S.D.M.). Statistical significance was analyzed using Student’s t-test (Systat Inc., Evanston, Ill., U.S.A.). p values less than 0.05 were considered statistically significant.

RESULTS Effects of Laminaria japonica Extract on the Blood Glucose Level in Streptozotocin-Induced Diabetic Rats The blood glucose level was significantly increased in the rats from the third day of streptozotocin injection, and reached 3—4 fold of the control group on the 5th day. The LJE supplement itself did not affect the blood glucose level in normal rats. However, blood glucose level in LJE (100 mg/kg)-pretreated diabetic rats was significantly lower than that in the diabetic group as shown in Fig. 1. Since we found that oral administration of a low concentration (50 mg/kg) of the extract had no significant effect on the blood glucose level in rats treated with streptozotocin and that a high concentration of the extract itself (200 mg/kg body weight) caused diarrhea in the rats (data not shown), we selected the concentration of 100 mg/kg body weight for the experiments on hepatic oxidative stress and xanthine oxidase.

Effects of Laminaria japonica Extract on Oxidative Stress in the Diabetic Rats Streptozotocin significantly increased the level of hepatic lipid peroxidation in diabetic rats on the 5th day and maintained it for 3 to 4 d (Fig. 2). Thereafter, lipid peroxidation was reduced in accordance with the reduction of liver weight (data not shown). Therefore, we examined the effect of LJE on hepatic lipid peroxide level on the 5th day following streptozotocin injection. As depicted in Fig. 2, pretreatment with LJE significantly suppressed the streptozotocin-induced lipid peroxidation in the rats.

Since GSH plays a major role in the protection of cells and tissue structures from oxidative stress, we also examined any alterations in GSH content. In fact, streptozotocin treatment significantly decreased the level of GSH, and the activities of hepatic GSH peroxidase and GSH reductase. The pre-treatment with LJE significantly prevented those alterations as shown in Table 1.

Preventive Effect of Laminaria japonica Extract on the Increased Activity and Type Conversion of Xanthine Oxi-
dase in the Diabetic Rat Liver  Since hepatic xanthine oxidase has been known as a major source of reactive oxygen species (ROS) generation in the pathogenesis of diabetic complications, we also examined whether the preventive effect of LJE on streptozotocin-induced hyperglycemia and oxidative stress is mediated through inhibition of XD/XO activity and type conversion. As summarized in Table 2, XO activity was similar in groups both untreated and those treated with LJE alone. Similarly, the type conversion ratio of XO/(XD+XO) was not changed in either the control or the group treated with LJE alone. In contrast, streptozotocin treatment significantly enhanced both hepatic XO activity and the type conversion ratio of XO/(XO+XD) in rat liver, which was significantly blocked by the pretreatment with LJE.

DISCUSSION  It is increasingly reported that oxidative stress plays an important role in diabetes, not only in experimental diabetes induced by streptozotocin but in human patients. Lipid peroxide-mediated tissue damage has been observed in the development of both type 1 and type 2 diabetes.25) In addition, treatment with antioxidant has lowered plasma glucose level in streptozotocin-induced diabetic rats.26) Similarly, our present results that water extract of Laminaria japonica suppressed the hyperglycemia and hepatic oxidative stress in streptozotocin-induced diabetic rats also support the role of such stress in a hyperglycemic condition of diabetes. In conjunction with the report that Laminaria japonica has antioxidant effects in diabetic rats,44) the present results suggest that the anti-hyperglycemic effect of Laminaria japonica may be mediated through its scavenging actions against ROS which cause hepatic injury and subsequent increase in blood glucose level. However, we do not exclude the possibility that the anti-hyperglycemic effect of LJE was mediated through its protective effect on the pancreatic cells from the action of streptozotocin, considering the reports that streptozotocin is concentrated preferentially in pancreatic beta cells via the glucose transporter-2,27) where it causes beta cell damage.28—30)

GSH is the first line of a defense system against pro-oxidant status. The GSH level is regulated by the enzymes GSH reductase and peroxidase. GSH reductase converts oxidized glutathione to a reduced form, while GSH peroxidase catalyzes the reduction of hydrogen peroxide in the presence of GSH to form water and oxidized glutathione. The decrease in GSH levels in liver during diabetes is probably due to its increased utilization by the hepatic cells. This may be due to the attempt by the hepatocytes to counteract the increased formation of lipid peroxides. The hepatic superoxide dismutase and catalase activities as other free radical scavenging system were not altered by streptozotocin (data not shown). The result that pretreatment with LJE significantly restored the GSH levels, GSH reductase and peroxidase activities indicates that LJE may either scavenge the ROS produced by STZ or enhance the utilization efficiency of GSH, thereby reduce the levels of lipid peroxidation in the diabetic rat liver.

Among various enzymes known to generate ROS, xanthine oxidase has recently been documented as a biological source to produce superoxide radicals which play important roles in the development of diabetic complications.9) In healthy volunteers, XO exists almost entirely (about 90%) in

<table>
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<tr>
<th>Table 1</th>
<th>Effects of Laminaria japonica Aqueous Extract on Hepatic GSH Content, GSH Reductase and GSH Peroxidase Activities</th>
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<tbody>
<tr>
<td>GSH (µmol/g of tissue)</td>
<td>GSH reductase (nmol/mg protein/min)</td>
</tr>
<tr>
<td>Control</td>
<td>6.83±0.51</td>
</tr>
<tr>
<td>LJE</td>
<td>7.04±0.49</td>
</tr>
<tr>
<td>STZ</td>
<td>5.04±0.68**</td>
</tr>
<tr>
<td>STZ+LJE</td>
<td>6.72±0.70***</td>
</tr>
</tbody>
</table>

Values are mean±S.D.M. of ten experiments. *p<0.05, **p<0.01 compared to control. # p<0.05, ## p<0.01 compared to untreated diabetic group.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Effects of Laminaria japonica Aqueous Extract on Hepatic Xanthine Oxidase Activity and Type Conversion in Streptozotocin Treated Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>XD (nmol/mg protein/min)</td>
<td>XO (nmol/mg protein/min)</td>
</tr>
<tr>
<td>Control</td>
<td>0.439±0.041</td>
</tr>
<tr>
<td>LJE</td>
<td>0.433±0.038</td>
</tr>
<tr>
<td>STZ</td>
<td>0.347±0.038**</td>
</tr>
<tr>
<td>STZ+LJE</td>
<td>0.408±0.035*</td>
</tr>
</tbody>
</table>

The type conversion ratio is represented as XO/XD×XO. Values are mean±S.D.M. of ten experiments. *p<0.01 compared to control. # p<0.05 compared to untreated diabetic group.
dehydrogenase form using NAD$^+$ as an electron acceptor that does not produce superoxide anion radicals. The clinical significance of XO in the oxidative stress of diabetes has been emphasized by a study in which treatment with allopurinol, a xanthine oxidase inhibitor, blocked the increase in plasma lipid peroxide levels in type 1 diabetic patients.\(^9\) More importantly, it has also been reported that the vascular xanthine oxidase originates in the liver of diabetic rats.\(^9\) In fact, the liver and intestine are the tissues that express the highest activity of the enzyme.\(^10\) Taken together, our results that LJE significantly reduced xanthine oxidase activity and type conversion of the enzyme in diabetic rat liver (Table 2) further suggest the clinical significance of LJE in the prevention of late-onset vascular complications of diabetes.

In conclusion, our results clearly showed that LJE significantly suppressed hyperglycemia and oxidative stress in streptozotocin-induced diabetes. In addition, the effects of LJE seem to be mediated through GSH content, the inhibition of xanthine oxidase activity and type conversion. The results further suggest that *Laminaria japonica* may be a valuable resource for lowering the hyperglycemia and for preventing vascular complications in diabetes mellitus.

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**REFERENCES**