Ischemia-reperfusion (I/R) injury of the intestine is an important factor associated with high rates of morbidity and mortality. Interruption of blood supply results in ischemic injury, which rapidly damages metabolically active tissues. Paradoxically, restoration of blood flow to the ischemic tissue initiates a cascade of events that may lead to additional cell injury known as reperfusion injury. This reperfusion damage frequently exceeds the damage caused by the original ischemic insult. The intestine is composed of labile cells that are easily injured by episodes of ischemia. Subsequent reperfusion of the intestine results in further damage to the mucosa.

Intestinal I/R is a common clinical problem in the settings of severe burns, circulatory shock and strangulation ileus. Furthermore, intestinal I/R injury is a serious medical problem often necessitating surgical intervention. The intestinal mucosa is damaged structurally and functionally after intestinal I/R, and intestinal mucosa damage induces local production of inflammatory cytokines. Intestinal I/R damages remote organs, including the lung, liver and kidney, as well as the intestine, and promotes multi-organ failure.

It has been reported that feeding condition has an effect on intestinal I/R injury. Thirteen-hour fasting before intestinal I/R results in more serious intestinal I/R injury than that in the case of 2-h fasting before intestinal I/R. In recent years, nutritional manipulation via the enteral route has been demonstrated to modulate dysfunctional inflammatory responses by preserving gut barrier function and immunity. It has been shown that enteral feeding before ischemic insults is beneficial for reducing organ injury and improving survival after intestinal I/R. In one of those studies, the authors used a standard complex enteral diet and they suggested that it is important to find new nutrient formulas. Since reactive oxygen species are responsible for intestinal I/R injury, we focused on a dietary polyphenol, the soy isoflavone genistein. Genistein has a wide spectrum of biochemical and pharmacological activities. However, the possibility of a protective effect of genistein as enteral nutrition on I/R injury has not been investigated. We therefore investigated the protective effect of genistein on oxidative injury using intestinal I/R model rats. We found that genistein, which has combined antioxidant activity from radical scavenging, xanthine oxidase inhibition and chain-breaking effects, exhibits a protective effect on intestinal I/R injury. The results suggest that genistein, a soy isoflavone, has the possibility as a new nutrient formula of enteral feeding.

Key words  genistein; ischemia-reperfusion; antioxidant; intestine; nutrition
MATERIALS AND METHODS

Chemicals Genistein, 2,2'-azobis(4-methoxy-2,4-dimethyl-valeronitrile) (MeO-AMVN) and 2,6-di-tert-butyl-4-methyl-phenol (BHT) were purchased from Wako (Osaka, Japan). Xanthine oxidase was purchased from Nacalai Tesque (Kyoto, Japan). Hypoxanthine was purchased from Sigma (St. Louis, MO, U.S.A.). 4,4-Difluoro-5-(4-phenyl-1,3-buta-diaryl)-4-bora-3a,4a-diaza-s-indacene-3-propionic acid sac-cinimidyl ester (BODIPY) was purchased from Invitrogen (Carlsbad, CA, U.S.A.). 2-Methyl-6-p-methoxyphenylethyl-nylimidazopyrazynone (MPEC) was purchased from ATTO Corp. (Osaka, Japan). Diethylretetramine-N,N',N'',N'''-penta-acetic acid (DTPA) was purchased from DOJINDO (Kumamoto, Japan). All other reagents were of the highest grade available and used without further purification.

Animals Male Wistar rats, aged 6 weeks (160—180 g in weight), were obtained from Jla (Tokyo, Japan). All rats were housed in stainless-steel cages. The housing conditions were the same as those described previously.[15] For the purpose of acclimation, the rats were allowed free access to food and water for at least 1 week. The experimental protocols were reviewed and approved by the Hokkaido University Animal Care Committee in accordance with the “Guide for the Care and Use of Laboratory Animals.”

Measurement of Superoxide Anion Scavenging Activity Superoxide anion (O_2^-) was generated by reaction between hypoxanthine and xanthine oxidase.[10] For measurement of superoxide anion, we used MPEC to emit oxidation. The compound for evaluation was dissolved with dimethyl sulfoxide. Xanthine oxidase and hypoxanthine were prepared with 0.1 M phosphate buffer (0.1 M–KH_2PO_4–NaOH (pH 7.5) and 0.05 mM ethylene diamine tetraacetic acid). The composition of the reaction solution for the superoxide anion scavenging activity test was 10 µl of the test sample, 10 µl of 300 µM MPEC, 170 µl of phosphate buffer, 60 µl of xanthine oxidase-phosphate buffer (0.1 units/ml), and 50 µl of hypoxanthine-phosphate buffer (0.72 mM).

The reaction to generate superoxide anion was initiated by adding hypoxanthine. Fifty microliters of the reaction solution was poured into a 384-well plate (Becton Dickinson, Franklin Lakes, NJ, U.S.A.) and light emission of 2-methyl-6-p-methoxyphenylethylimidazopyrazynone was measured by a Wallac 1420 ARVOx multispectral counter (Perkin Elmer, Wellesley, MA, U.S.A.). The 50% inhibitory concentration value was determined from the concentration-inhibition of light emission curve calculated by using Origin® (version 6.1J). A formula fitting logistically was expressed as follows:

\[ Y = (A_0 - A_1)/(1 + ((x + x_{50})^P)) + A_2 \]

where \( A_1 \) is the initial value, \( A_2 \) is the final value, \( x \) is concentration, \( x_{50} \) is the IC_{50} value and \( P \) is power.

Measurement of Hydroxyl Radical Scavenging Activity The method for measurement is based on the generation of HO· by Fenton reaction and the determination of HO· by chemiluminescence.[10] For the Fenton reaction, H_2O_2 solution (1 mM) was prepared by diluting hydrogen peroxide solution with 25 mM imidazole–nitrite solution (pH 7.0), ferrous sulfate solution (1 mM) was prepared by dissolving FeSO_4·7H_2O in distilled water, and DTPA solution (1 mM) was prepared by distilled water. As a chemiluminescent reagent, luminol solution (1 mM) was prepared by dissolving luminol in 200 mM borate buffer (pH 9.5). The reaction solution was composed of 0.16 mM ferrous sulfate, 0.16 mM DTPA, 0.25 mM hydrogen peroxide, and 0.16 mM luminol with or without genistein (final concentrations of 0.1, 1, 10, 100, 1000, 10000 µM). The ferrous iron (Fe^{2+})–H_2O_2–luminol chemiluminescence signal profile was obtained by using the multilabel counter Wallac 1420 ARVOSx (Perkin Elmer, Wellesley, MA, U.S.A.). The results are expressed as inhibition, representing the percentage of inhibition of luminol chemiluminescence with respect to that occurring in a control:

\[ \text{inhibition rate} (\%) = \frac{(AUC_{\text{control}} - AUC_{\text{sample}})}{AUC_{\text{control}}} \times 100 \]

where \( AUC_{\text{control}} \) and \( AUC_{\text{sample}} \) represent the area under the curve (AUC) of luminol chemiluminescence kinetics in the control and that in samples, respectively.

Measurement of Radical Chain-Breaking Activity The assay was carried out as described in a previous report with some modification.[8] Stock solutions of the fluorescent lipophilic probe BODIPY were prepared in dimethyl sulfoxide and stored under nitrogen at −70 °C. For BODIPY incorporation, 25 µl of the BODIPY stock solution (2 mM) was diluted 100-fold with phosphate buffer saline (PBS). Then aliquots of 50 µl were added to 50 µl of rat plasma, diluted to 350 µl of PBS with or without tested compounds (final concentrations of 0.1, 1, 10, 100, 1000, 10000 µM), vortexed for 20 s at the lowest speed, and incubated under aerobic conditions for 10 min at 37 °C. The final volume was adjusted to 480 µl with PBS, yielding BODIPY at a final concentration of 2 µM. MeO-AMVN was dissolved in acetonitrile immediately before use, and 20 µl of the solution was added to the sample at a final concentration of 2 mM. Then aliquots of 200 µl were transferred to a 96-microwell plate, and the lipid oxidation kinetics was monitored by measuring the green fluorescence (\( \lambda_{ex} = 485, \lambda_{em} = 535 \) nm; cycle time of 10 min for 18 cycles) of the oxidation product of BODIPY. Light emission was measured with the multilabel counter Wallac 1420 ARVOSx (Perkin Elmer, Wellesley, MA, U.S.A.). The results are expressed as total antioxidant performance (TAP) values, representing the percentage of inhibition of BODIPY oxidation in human plasma with respect to that occurring in a control sample:

\[ \text{TAP} = \frac{(AUC_{\text{control}} - AUC_{\text{plasma}})}{AUC_{\text{control}}} \times 100 \]

where \( AUC_{\text{control}} \) and \( AUC_{\text{sample}} \) represent the AUC of BODIPY oxidation kinetics in the control samples and plasma samples, respectively. Control samples were prepared using PBS.

Intestinal I/R Model Surgical procedures were carried out as described in a previous report with some modification.[21] Wistar rats were anesthetized with sodium pentobarbital (40 mg/kg weight, intraperitoneally (i.p.)). The rats were fixed after the operation. A small midline incision was made in the abdomen. A 5-cm-long loop of the jejunum was identified and ligated at both ends. Through a midline laparotomy, the superior mesenteric artery (SMA) was isolated and a bulldog arterial clamp was applied at the aortic origin. The abdomen was then covered with a sterile plastic wrap. After 30 min of intestinal ischemia, the arterial clamp was removed. Five hundred microliters of genistein solution (1 mM)
was administered directly into the loops 1 h before the induction of ischemia.

**Histological Examination** Histological samples were sent to the Sapporo General Pathology Laboratory Co., Ltd. Samples of the small intestine were harvested after reperfusion and immediately fixed in 10% buffered formalin. The fixed tissue was embedded in paraffin, sectioned to 4 μm in thickness, and stained with hematoxylin–eosin.

**Evaluation of Changes in Vascular Permeability** Extravasation of Evans blue dye into tissue was used as an index of increased vascular permeability. Evans blue dye (20 mg/kg) was injected (1 ml/kg) through the jugular vein 5 min prior to reperfusion of the ischemic artery. Sixty minutes after reperfusion, fragments of the loop were cut and allowed to dry in a petri dish for 24 h at 37 °C. The dry weight of the tissue was calculated and Evans blue dye was extracted using 4 ml of formamide (24 h at 37 °C). The dye concentration of the samples was measured by spectrophotometry at 620 nm using JASCO V-630 BIO (Tokyo, Japan). Results are presented as the amount of Evans blue in μg per 100 mg of tissue.

**Measurement of Lipid Peroxide** After reperfusion, the 10-cm-long portion of the jejunum and ileum was excised to measure protein and lipid peroxide contents. The tissue sample was cleansed in ice-cold saline and homogenized in 2.0 ml of saline using a Potter–Elvehjem homogenizer with 20 strokes. Lipid peroxide is not a single constituent. Thus, the amount of lipid peroxide was determined as that of malondialdehyde (MDA) by Yagi’s method (1979) with some modification. The fixed tissue was cleansed in ice-cold saline and homogenized in 2.0 ml of saline using a Potter–Elvehjem homogenizer with 20 strokes. Lipid peroxide is not a single constituent. Thus, the amount of lipid peroxide was determined as that of malondialdehyde (MDA) by Yagi’s method (1979) with some modification.

**Data Analysis** Nonlinear regression analysis was performed using Origin® (version 6.1J). Student’s t-test was used to determine the significance of differences between two group means. Statistical significance among means of more than two groups (Figs. 4, 5) was determined by one-way analysis of variance (ANOVA) followed by Turkey’s test. A value of p<0.05 was considered significant.

**RESULTS**

**Inhibition of Xanthine Oxidase-Induced Light Emission**

Superoxide anion scavenging activity was measured using an MPEC reaction kit. Superoxide anions were generated by xanthine/xanthine oxidase. The experimental protocol is described in Materials and Methods. Each point represents the mean with S.D. of 3 independent experiments.

**Inhibition of Fenton Reaction-Induced Chemiluminescence**

Hydroxyl radical-scavenging activity of genistein was measured by a method based on the generation of HO· by Fenton reaction. The experimental protocol is described in Materials and Methods. Each point represents the mean with S.D. of 3 independent experiments.

**Inhibitory Effect of Genistein on BODIPY Oxidation**

Since the influence of free radical-mediated oxidation is amplified because it is proceeded by a chain mechanism, the role of chain-breaking activity is important as well as radical scavenger activity. Recently, Beretta et al. reported a method that enables specific measurements of chain-breaking activities of tested compounds using BODIPY. We therefore investigated the chain-breaking activity of genistein using BODIPY. Genistein inhibited BODIPY oxidation, and the effect was concentration-dependent. Thus, the TAP value
of genistein also increased in a concentration-dependent manner (Fig. 3).

Protective Effect of Genistein on Intestinal I/R Injury

We confirmed in vivo antioxidant activity of genistein using a rat I/R injury model. Since vascular permeability has been shown to be significantly higher in rats with I/R than in sham-operated rats, elevated vascular permeability is also one of the indicators of I/R injury. We therefore investigated the effect of genistein on increased vascular permeability, as assessed by reduction in the level of Evans blue dye. Elevation of vascular permeability by intestinal I/R was significantly attenuated by treatment with genistein (Fig. 4).

Next, we investigated the mechanism of the inhibitory effect of genistein on lipid peroxidation after intestinal I/R. Lipid peroxidation is an integral process in the oxidation of unsaturated fatty acids via a radical chain reaction, and lipid peroxide is accumulated in the small intestine by I/R injury. The level of lipid peroxidation in the I/R group was significantly increased compared with that in the sham-operated group. On the other hand, the increased level of lipid peroxidation in the intestine tended to be reduced by administration of genistein (Fig. 5).

We additionally investigated the level of injury by intestinal I/R histologically (Fig. 6). Loss of villi and damage to the crypt in the I/R group were clearly observed compared with the sham-operated group (Figs. 6A, B). On the other hand, both loss of villi and damage to the crypt were attenuated by pretreatment with genistein (Fig. 6C). We confirmed that genistein administered into the rat intestinal loop in the sham group did not influence the Evans blue dye leak amount, MDA and histological appearance (data not shown).

DISCUSSION

I/R injury of the intestine is a significant problem in abdominal aortic aneurysm surgery, small bowel transplantation, cardiopulmonary bypass, strangulated hernias, and neonatal necrotizing enterocolitis. It can also occur as a consequence of collapse of systemic circulation, as in hypovolemic and septic shock. This is thought to be derived from oxidative injury. It is also associated with high rates of morbidity and mortality. Several studies have demonstrated an alteration of absorptive function of the intestine following intestinal I/R injury. This could lead to deficient absorption of nutrients. In severe cases such as infarction of the bowel, short-bowel syndrome can occur.

Enteral nutrition has been shown to reduce death rate and remote organ injury using an animal model. Demirkiran et al. reported the protective effect of glutamine pretreatment against intestinal I/R. Fukushima et al. demonstrated that enteral feeding maintains mucosal immunity and resistance to infections. Recently, they have indicated that it is important to be aware that other nutrient formulas might improve survival after intestinal I/R. Many studies have focused on the antioxidant activities of flavonoids. Genistein is the most popular and attractive soy isoflavone in this regard. The protective effect of genistein on I/R injury such as that in the hippocampus has been also studied using an animal model. Moreover, it has been reported that genistein-concentrate enteral diet may have an anticancer effect on colon carcinogenesis. However, the possibility of genistein as enteral nutrition for prevention of I/R injury has not been investigated. Thus, we investigated the protective effect of genistein on intestinal I/R injury.

In the first part of this study, the antioxidant activities of genistein were evaluated using an in vitro system. The super-
oxide radical anion appears to play a central role since other ROS are formed in reaction sequences starting with the superoxide radical anion. Xanthine/xanthine oxidase is also a main source of ROS.26) Moreover, intestinal mucosa is one of the richest sources of xanthine oxidase. In this study, we therefore used a xanthine/xanthine oxidase system to generate superoxide anions. Genistein inhibited the light emission induced by xanthine oxidase in a concentration-dependent manner (Fig. 1). We also investigated the hydroxyl radical-scavenging activity of genistein since hydroxyl radical production plays a significant role in the initiation of lipid peroxidation. The results showed that genistein exhibited hydroxyl radical-scavenging activities (Fig. 2). Since the influence of free radical-mediated oxidation is amplified because it is proceeded by a chain mechanism, the role of chain-breaking activity is important as well as radical scavenger activity.27) We therefore investigated the chain-breaking activity of genistein using BODIPY. Genistein exhibits chain-breaking activity as well as radical scavenger activity (Fig. 3). We consider that assays which we use in this study are appropriate for the evaluation of antioxidant activities since we have already reported them.36) The results of these assays are very significant findings from the point of view of measuring the different point of each antioxidant activity and clarifying the detailed antioxidant mechanism of genistein. We have evaluated that the inhibitory effect of polyphenols such as rutin, chlorogenic acid, caffeic acid and ferulic acid that have antioxidant activities on xanthine oxidase-induced light emission, fenton-reaction-induced chemiluminescence and BODIPY oxidation.23,36,37) In these reports, we reported that the IC_{50} values of rutin, chlorogenic acid, caffeic acid and ferulic acid on xanthine-oxidase induced light emission by MPEC assay were about 71.7, 41.0, 10.1 and 6614 μM, respectively.23,36,37) It is suggested that the effect of genistein on the inhibition of xanthine oxidase would not be so strong compared with other polyphenols from the point that the IC_{50} value of genistein in this study was about 700 μM (Fig. 1). We also investigated the hydroxyl radical scavenging activity by luminol chemiluminescence. As well as the inhibitory effect of some polyphenols on xanthine oxidase-induced light emission, genistein did not show the inhibitory effect of fenton-reaction-induced chemiluminescence strongly (Fig. 2). The TAP value of genistein on the inhibition of radical chain reaction was not superior to other polyphenols. The average ingestion amount of genistein in humans is about 15—22 mg/d and about 30 mg/d are recommended amount of it.38,39) We set a dose of genistein into the intestinal loop 1 mM (135 μg) in this study. We considered that the appropriate concentration of genistein would be 1 mM since sufficient antioxidant activity of genistein at a concentration of more than 1 mM was demonstrated by the MPEC assay (Fig. 1), luminol chemiluminescence (Fig. 2) and TAP values (Fig. 3) described above. Zhou et al. reported that the bioavailability of genistein in rats was about 20—30%.40) It is highly likely that genistein of this concentration is in the intestine. In this study, we focused on the protective effect of genistein in the intestine.

We then confirmed the protective effect of genistein on intestinal I/R injury using I/R injury model rats. We have already reported that other antioxidants exhibit antioxidant ac-
tivities and attenuate intestinal I/R injury.\textsuperscript{23,36,41} Reperfusion of the ischemic intestine results in an increase in vascular permeability to Evans blue dye (Fig. 4). Extravasation of Evans blue was used as a marker of changes in capillary permeability and tissue edema.\textsuperscript{22} Elevation of vascular permeability by intestinal I/R was attenuated by treatment with genistein (Fig. 4). We next investigated the direct effect of genistein on I/R injury by measuring the amount of MDA, an index of lipid peroxidation (Fig. 5). Genistein did not show the significant protective effect on lipid peroxidation after intestinal I/R but the increased level of lipid peroxidation in the intestine tended to be reduced by administration of genistein. Highly reactive free radicals, such as peroxyl radicals involved in auto-oxidation of lipoproteins and of biological membranes, are responsible for microvascular damage. We consider that genistein showed the protective effect from microvascular damage rather than the inhibitory effect of lipid peroxidation. We additionally observed changes in the intestinal tissue after I/R microscopically (Fig. 6). Damage to the crypt and shortening of the villi in the intestine are typical histological findings after I/R described previously.\textsuperscript{25} Pretreatment with genistein attenuated both of these in intestinal I/R injury. These results of histological appearance were consistent with the amount of that of Evans blue dye leakage after intestinal I/R. The protective effect of genistein on membrane structure was strong. The reason of this result remains obscure in the present study. Further investigations are needed to clarify the mechanism of the protective effect of genistein on membrane structure.

From the result of this study, we consider that genistein would attenuate the increased vascular permeability by intestinal I/R rather than prevent the increased MDA levels. Intestinal I/R injury is magnified by many events, such as oxygen free radical formation, actions of inflammatory cytokines, complement activation, and neutrophil infiltration at the site of injury.\textsuperscript{41} In addition to radical-scavenging activity and chain-breaking activity, genistein exhibits many kinds of anti-inflammatory effects and affects complement factors.\textsuperscript{43—45} Liang \textit{et al.} have reported that genistein attenuated oxidative stress and suggested that the effect was derived from the inhibition of apoptosis.\textsuperscript{36} So we would like to measure myeloperoxidase activity, glutathione, xanthine oxidase and factors associated with apoptosis after intestinal I/R at an early date. Further investigations to establish the evidence of the effectiveness of genistein as an enteral nutrition in clinical settings are in progress.

An excess of ROS induces oxidative damage in vulnerable targets such as membrane unsaturated fatty acids, protein thiols, and DNA bases.\textsuperscript{46} In addition to I/R injury, ROS contribute to the development of various chronic diseases such as cancer, neurodegenerative diseases, and cardiovascular diseases.\textsuperscript{47,48} Much interest has been shown in the discovery of new antioxidants in recent years due to their potential applications in the treatment of diseases induced by ROS. For the prevention of these diseases, an approach focusing on the health aspects of natural antioxidants should be beneficial.\textsuperscript{23,36,37,41} In order to develop new functional foods, it is important to select a suitable ingredient that has a scientific background. The results of this study should provide a scientific background of the usefulness of genistein as a functional food ingredient.

In summary, we have found that genistein, which has combined antioxidant activity from radical scavenging, xanthine oxidase inhibition and chain-breaking effects, exhibits a protective effect on I/R injury in the rat small intestine. These results suggested that genistein would be a new enteral nutrition against oxidative injury. We would like to clarify the correlation with oxidative injury and the concentration of antioxidant and apply genistein to the enteral nutrient. Further investigations are needed to improve the availability of genistein, a familiar soybean isoflavone, as an enteral nutrition in clinical settings.

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**REFERENCES**