The seeds of the Japanese horse chestnut (Aesculus turbinata BLUME) have been utilized as an emergency provisions since ancient times. Nowadays, after treating them with wood ashes to remove bitter saponins, edible seeds are being used as ingredients of traditional foods like rice balls and rice cake in Japan. The biological activities of the seed shell ingredients of the Japanese horse chestnut, which have been waste products for the preparation of edible seeds, have become a focus of study [17, 18, 26]. Recently, Ogawa et al. have reported that seed shells contain higher levels of polyphenolic antioxidants [26]. The predominant polyphenolic compounds from seed shells of the Japanese horse chestnut are highly polymeric proanthocyanidins having doubly-linked A-type interflavan linkages as well as singly-linked B-type bonds without gallic acid esterified to them [26].

Methotrexate (MTX), a structural analogue of folic acids, is widely used as a chemotherapeutic drug in the treatment of leukemia and other malignancies [15]. Being a high affinity inhibitor of dihydrofolate reductase, MTX is a pro-oxidant compound that causes depletion of the dihydrofolate pool and directly affects the synthesis of thymidilate, suppressing DNA synthesis [15, 19]. Since the cytotoxic effect of MTX is not selective for cancer cells, it also affects normal tissue that have a high rate of proliferation, such as the gut mucosa. Thus, the efficacy of MTX is often limited by severe side effects such as intestinal injury [1, 7, 15, 31]. MTX-induced intestinal injury results in malabsorption syndrome through serious malabsorption of nutrients and diarrhea [1, 28].

Reactive oxygen species (ROS) are implicated in the pathogenesis of the MTX-induced gastrointestinal mucosal injury [11]. It was also demonstrated that MTX caused a significant reduction in antioxidant protein and enzyme levels and an increase in levels of oxidant markers such as malondialdehyde (MDA) [12, 16, 25, 29]. Therefore, in order to minimize side-effects of MTX in patients, the studies focused on antioxidants [12, 16, 25, 29, 32].

Many polyphenols are known to be antioxidants, and the possibility exists that they protect against oxidative damage by directly neutralizing reactive oxidants [8, 14, 21, 22, 24, 30]. However, to our knowledge, there are no reports showing the protective effects of polyphenols from seed shells of Japanese horse chestnut (JHP) against oxidative stress-related pathologic conditions in vivo. The purpose of this study is to evaluate the effects of JHP on MTX-induced intestinal injury in rats through its antioxidant properties.

The seeds of the Japanese horse chestnut (Aesculus turbinata BLUME) were collected from the forests of northern Hyogo Prefecture in Japan and identified as described earlier [17]. Diaion HP-20 and Chromatorex ODS 1024T for column chromatography were obtained by Nippon Rensui (Tokyo, Japan) and Fuji Silysia (Kasugai, Japan), respectively. Polyphenolic compounds were extracted and separated according to our earlier-reported procedures [26]. Briefly, seed shells of the Japanese horse chestnut (20 g as fresh weight) were ground into powder and refluxed by boiling for 2 hr in 400 ml of water. The extracts were purified by column chromatography on Diaion HP-20 and subsequent Chromatorex ODS 1024T to recover the fraction of polyphenolic compounds from seed shells of the Japanese horse chestnut. In this result, 0.86 g of the separated polyphenolic compounds (JHP) were obtained.

Wistar strain male rats weighing 220.67 ± 3.19 (mean ± SE) g were obtained from the Institute of Animal Reproduc-
tion (Kasumigaura, Japan). The animals were maintained at a controlled temperature of 22 ± 2°C with a 12:12-hr light/dark cycle (light cycle, 7:00–19:00), and were given standard chow (CE-2, Nihon Clea, Tokyo, Japan). The use of these animals and the procedures used with them were approved by the Animal Research Committee at Tottori University.

A total of 36 animals were divided into 6 groups containing 6 rats as follows. Group I (saline plus saline treated control group): Rats in group I were orally administered saline for 7 days. They received the equivalent volumes of saline intraperitoneally instead of MTX once daily for 4 days on the 3rd - 7th day. Group II and III (JHP plus saline treated control group): Animals in group II and III were orally administered JHP (group II, 100 mg/kg; group III, 300 mg/kg) for 7 days. They received intraperitoneally saline once daily for 4 days on the 3rd - 7th day. Group IV (saline plus MTX treated control group): Animals in group IV were orally administered saline for 7 days. They received intraperitoneally MTX (1.25 mg/kg) once daily for 4 days on the 3rd - 7th day. Group V and VI (JHP plus MTX treated group): Animals in group V and VI were orally administered JHP (group V, 100 mg/kg; group VI, 300 mg/kg) for 7 days. They received intraperitoneally MTX (1.25 mg/kg) once daily for 4 days on the 3rd - 7th day.

Small intestinal tissues were fixed in 10% neutral buffered formalin, processed for histological examination by the conventional methods, and stained with hematoxylin and eosin (HE). Those conducting the histopathological examinations were blinded to the study treatments. An overall score of intestinal damage severity was semiquantitatively assessed by the method of Vardi et al. [32] as follows: villus fusion, desquamation of surface enterocytes, crypt loss, disruption or distortion of crypt cells, inflammatory infiltrate in the lamina propria and lymphocytes and dilation of lymphatics and capillaries. The microscopic score of each tissue was calculated as the sum of the scores given to each criterion. Scores were given as 0 (normal), 1 (mild), 2 (moderate) and 3 (severe) for each criterion. Thus, the maximum score reached 18.

For immunohistochemistry, the following primary antibodies were used: Ki-67 antigen mouse monoclonal antibody (DakoCytomation, Tokyo, Japan). All sections were dewaxed, rehydrated, rinsed with 0.05 M tris-buffered saline (TBS; pH 7.6), treated with 3% hydrogen peroxide, and then rinsed again with TBS. Tissue sections for the detection of Ki-67 antigen were immersed in 0.01 M citrate buffer (pH 6.0) and autoclaved for 15 min at 121°C to retrieve antigen. Slides were incubated with primary antibody at 4°C overnight and, after rinsing with TBS, were treated with Simple Stain MAX-PO (M) (Nichirei, Tokyo, Japan) for 30 min at room temperature. They were then rinsed with TBS before being treated with a 3,3’-diaminobenzidine solution containing 0.01% hydrogen peroxide to facilitate a peroxidase colour reaction. After a further wash with TBS, the slides were counterstained with Mayer’s haematoxylin. A semiquantitative analysis was performed by counting the Ki-67 antigen-positive cells per field at ×400 magnification in the crypt cells with histometric analysis software (Olympus Co., Ltd., Tokyo, Japan). The mean number of such cells in 50 randomly selected fields in each small intestine was expressed as the number of Ki-67 antigen-positive cells.

Small intestine samples were dissected out and washed immediately with ice cold saline to remove as much blood as possible. The intestinal mucosal scrapings were homogenized with ice-cold RIPA buffer (5% w/v; Thermo Scientific, Rockford, IL, U.S.A.) using a the Polytron PT2100S homogenizer (Kinematica, Inc., New York, U.S.A.). The unbroken cells and cell debris were removed by centrifugation at 2,000 rpm at 4°C for 10 min, and clear supernatant was used for estimation of the activities of intestinal glutathione peroxidase (GSH-Px), intestinal MDA and GSH levels. Intestinal GSH-Px activity was determined in a coupled assay with glutathione reductase by measuring the rate of NADPH oxidation at 340 nm using H2O2 as the substrate [27] with a commercial kit (Northwest Life Science Specialties, Vancouver, BC, Canada). GSH-Px activity was expressed as U/g for intestinal mucosal tissue. The MDA levels were assayed for products of lipid peroxidation by monitoring thiobarbituric acid-reactive substance (TBARS) formation with commercial kit (Northwest Life Science Specialties, Vancouver, BC, Canada) as described previously [6, 9]. Tissue supernatant was mixed with acid reagent and 2-thiobarbituric acid reagent and incubated 60 min at 60°C. After cooling, the precipitate was removed by centrifugation. The absorbance of the supernatant was recorded at 532 nm. The values of MDA were expressed as nmol/g for intestinal mucosal tissue. Intestinal GSH content was determined using commercial kit (Northwest Life Science Specialties, Vancouver, BC, Canada) according to the method of Beutler et al. [5]. The formation of 5-thio-2-nitrobenzoate (TNB) is followed spectrophotometrically at 412 nm and the levels of GSH results were expressed as μmol GSH/g for intestinal mucosal tissue.

All data are expressed as means ± SE of all rats in each group. The results in each group were compared by one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test with statistical software (SSRI Co., Ltd., Tokyo, Japan). P<0.05 was considered to be statistically significant.

All rats in six groups showed no significant changes in clinical observation such as body weight loss, diarrhea or bloody stools. While the tissue sections of the rats in the group I-III (saline or JHP plus saline-treated group) showed normal intestinal structures, considerable changes were observed in the small intestine in group IV (saline plus MTX-treated group) (Fig. 1). In group IV, shortening of villus or fusion of villi, atrophy of surface epithelium, cystic dilation in crypts, crypt loss and prominent leukocyte infiltration were observed (Fig. 1). Cellular debris was observed in the lumen of the crypt. A decreased number of mitotic figures in crypts and goblet cells in villi were recognized. The histopathological changes in group V and VI (JHP 100
or 300 mg/kg plus MTX-treated groups) were alleviated, compared to those in group IV (Fig. 1). The total intestinal damage score in group V and VI was significantly reduced compared to group IV (Fig. 2). The number of Ki-67 antigen-positive cells was significantly decreased in group IV. The number of Ki-67 antigen-positive cells in group V and VI showed a tendency to be increased in comparison to group IV (Figs. 1 and 2).

Figure 3 shows the effects of JHP supplementation on the levels of intestinal GSH, GSH-Px and MDA. MTX induced a significant decrease in the levels of intestinal GSH and GSH-Px in group IV (saline plus MTX-treated group). JHP supplementation showed a tendency to restore the levels of GSH and GSH-Px activity in group V and VI (JHP 100 or 300 mg/kg plus MTX-treated groups). The intestinal MDA levels in group IV were higher than in group I (saline plus saline treated group). The level of MDA in group V and VI showed a tendency to be reduced compared to group IV.

MTX reportedly causes morphological intestinal injury associated with oxidative damages [12, 16, 20, 29]. Our results revealed that MTX induced severe morphological intestinal injury and increase in the level of lipid oxidation marker, MDA, and decrease in the levels of the antioxidant markers, GSH and GSH-Px, similarly to previous studies [12, 16, 25, 29]. JHP supplementation ameliorated the MTX-induced morphological intestinal injury and preserved intestinal MDA, GSH and GSH-Px levels. These findings suggested that the alleviative effect of JHP on intestinal damage was due to its antioxidant properties.

Previous studies suggested that lipid peroxidation was a contributing factor to the development of MTX-induced intestinal damage [12, 16, 25, 29]. It has been reported that polyphenol extracted from apple [8, 24], green tea [14, 22] and rhizome of Curcuma longa [30] could diminish lipid peroxidation. In the present study, parallel with these reports, the decrease in intestinal MDA level was observed in the JHP plus MTX-treated rats, but not in the saline plus MTX-treated rats. This result indicates that mechanism of alleviation of intestinal damages by oral administration of JHP is associated with decreasing lipid peroxidation.

GSH is the most important endogenous antioxidant in cells and has a direct antioxidant function by reacting with superoxide radicals, peroxyl radicals and singlet oxygen followed by the formation of oxidized glutathione (GS-SG) and other disulfides. Glutathione S-transferase (GST) and GSH-Px are GSH-dependent antioxidant enzymes [23].
MTX treatment depletes intestinal GSH levels significantly [25, 29, 32]. The significant reduction in GSH levels promoted by MTX, represents an alteration in the cellular redox state, suggesting that cells could be more sensitive to ROS and thus lead to a reduction in the effectiveness of the antioxidant enzyme defense system [2]. Our results also revealed that MTX treatment caused a significant decrease in intestinal GSH levels similarly to previous studies [25, 29, 32]. However, due to its antioxidant activity, JHP administration impeded the reduction in GSH level in the groups exposed to MTX.

Moskaug et al. [21] reported that dietary plant polyphenols modulate expression of gamma-glutamylcysteine synthetase in both cellular antioxidant defenses and detoxification of xenobiotics. This enzyme is rate limiting in the synthesis of GSH. A similar mechanism may preserve the level of intestinal GSH in the JHP plus MTX-treated group of this study.

JHP exhibited potent antioxidative activities comparable to monomeric (+)-catechin and (–)-epicatechin, or more efficacious than those monomers [26]. The predominant polyphenolic compounds from seed shells of the Japanese horse chestnut are polymeric proanthocyanidins [26], which have been shown to serve as one of free radical scavengers and antioxidants both in vitro and in vivo [10]. In vivo studies have shown that grape seed proanthocyanidin extract is a better free radical scavenger and inhibitor of oxidative tissue damage than vitamin C, vitamin E succinate, vitamin C and vitamin E succinate combined, and β-carotene [3, 4]. Moreover, in vitro studies indicated that proanthocyanidins have specificity for the hydroxyl radical [4] in addition to the ability to non-competitively inhibit the activity of xanthione oxidase, a major generator of free radicals [13].

It was known that MTX inhibited crypt cell proliferation in small intestine [1, 19, 28]. In the present study, parallel with these reports, MTX induced a significant decrease of Ki-67 antigen positive rate in the present study.
other hand, JHP administration restored Ki-67 antigen positive rate lowered by MTX injection in the present study. The exact mechanism of restoration of cell proliferative activity by JHP remain unclear, it is likely that it is relative to an antioxidative effects of JHP.

In conclusion, our results indicated that the protective effect of JHP against MTX-induced intestinal damages via its antioxidative action. To our knowledge, this is the first report of the protective effect of JHP on oxidative damages in vivo. Our results support the potential use of JHP in clinical conditions where oxidative organ failure may be present.

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