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Scutellariae Radix enhances but Scutellariae Radix-containing Kampo formulas, Orengedokuto and San'oshashinto, prevent intestinal bleeding associated with indomethacin-induced enteropathy in mice

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A single subcutaneous injection of indomethacin (INDO) in mice induced enteropathy characterized as ulceration in small intestine, which was associated with the elevation of fecal hemoglobin (Hb) content and the decrease in Hb and total protein concentration in blood. Oral administration of Scutellariae Radix (SR) (500 mg/kg) enhanced fecal Hb excretion but did not affect the decrease in blood Hb and total protein concentration in INDO-treated mice. In contrast, administration of two SR-containing kampo formulas, Orengedokuto (OGT) and San'oshashinto (SST) at 1100 mg/kg, which contained nearly the same amounts of baicalin as SR extracts at 500 mg/kg, markedly suppressed INDO-induced intestinal bleeding and blood loss. Plasma INDO concentration was not changed by the administration of SR, OGT or SST. The present study suggests that OGT and SST are useful for limiting complications such as intestinal bleeding and blood loss associated with INDO-induced enteropathy. However, possible harmful effects of other SR-containing kampo formulas on INDO-induced enteropathy remain to be investigated.

Key words NSAIDs, small intestine, hemoglobin, bleeding, blood loss, baicalin.

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are extensively used as anti-pyretics and anti-inflammatory analgesics, although long-term ingestion of NSAIDs induces gastrointestinal side-effects such as ulceration in the stomach and duodenum.[1] In addition, recent investigations revealed that the ingestion of NSAIDs induces enteropathy characterized as ulceration in small intestine[2] and severe blood loss due to intestinal bleeding, which are relevant in the rheumatic patients taking NSAIDs.[3-5] The experimental studies have also confirmed that the administration of several types of NSAIDs can induce enteropathy with predominant damage in the small intestine accompanied by intestinal bleeding and blood loss.[6,7] Although the precise mechanism by which NSAIDs induce enteropathy and intestinal bleeding has not fully understood, early inflammatory responses upon NSAID administration such as the increased mucosal permeability and leukocyte infiltration are suggested to play important roles.[8] Therefore, pharmacological modulation of these inflammatory reactions induced by NSAIDs is expected to ameliorate NSAID-induced enteropathy, which may be accompanied by the attenuation of intestinal bleeding and blood loss.

There are reports indicating that the administration of Scutellariae Radix (roots of Scutellariae baicalensis George) (SR) or SR-containing kampo formulas ameliorates the inflammatory reactions and pathological changes during the experimental intestinal diseases.[9-12] In addition, flavonoids contained in SR such as baicalin and wogonin have been reported to exert the modulatory actions on inflammatory responses,[13-15] which can explain the amelioration of experimental intestinal diseases by SR or SR-containing kampo formulas.[9-12] Therefore, SR or SR-containing kampo formulas are also expected to modulate NSAID-induced intestinal injury. In addition, since there may be cases that kampo formulas are used with NSAIDs during the therapy for inflammatory diseases, the effects of kampo formulas on NSAID-induced enteropathy should be clarified. In the present study, SR and SR-containing kampo formulas, Orengedokuto (OGT) and San'oshashinto (SST), were evaluated for indomethacin-(INDO) induced intestinal bleeding and blood loss associated with NSAID-induced enteropathy in mice.

Material and Methods

Drugs. Lyophilized powder of water extracts of Scutellariae Radix (SR), Orengodokuto (OGT) and San'oshashinto (SST) were kindly provided from Tsumura & Co. (Tokyo, Japan). OGT and SST are composed of the herbal components listed in Table 1. Indomethacin (INDO) and dimethylsulfoxide (DMSO) were purchased from Wako Pure Chem (Osaka, Japan).

Animals and drug treatments. Male ddY mice at 5 weeks of age (SLC Japan, Shizuoka, Japan) were housed in plastic cages placed in an air-conditioned room (temperature at 23±2 ℃ and humidity at 55±5 %) under a 12 hr: 12 hr light-dark cycle; the light was turned on at 09.00 h. The animals were maintained under the above conditions at least for 1 wk prior to the experiments below. Overall experimental procedures were shown in schema 1. The extracts of SR,

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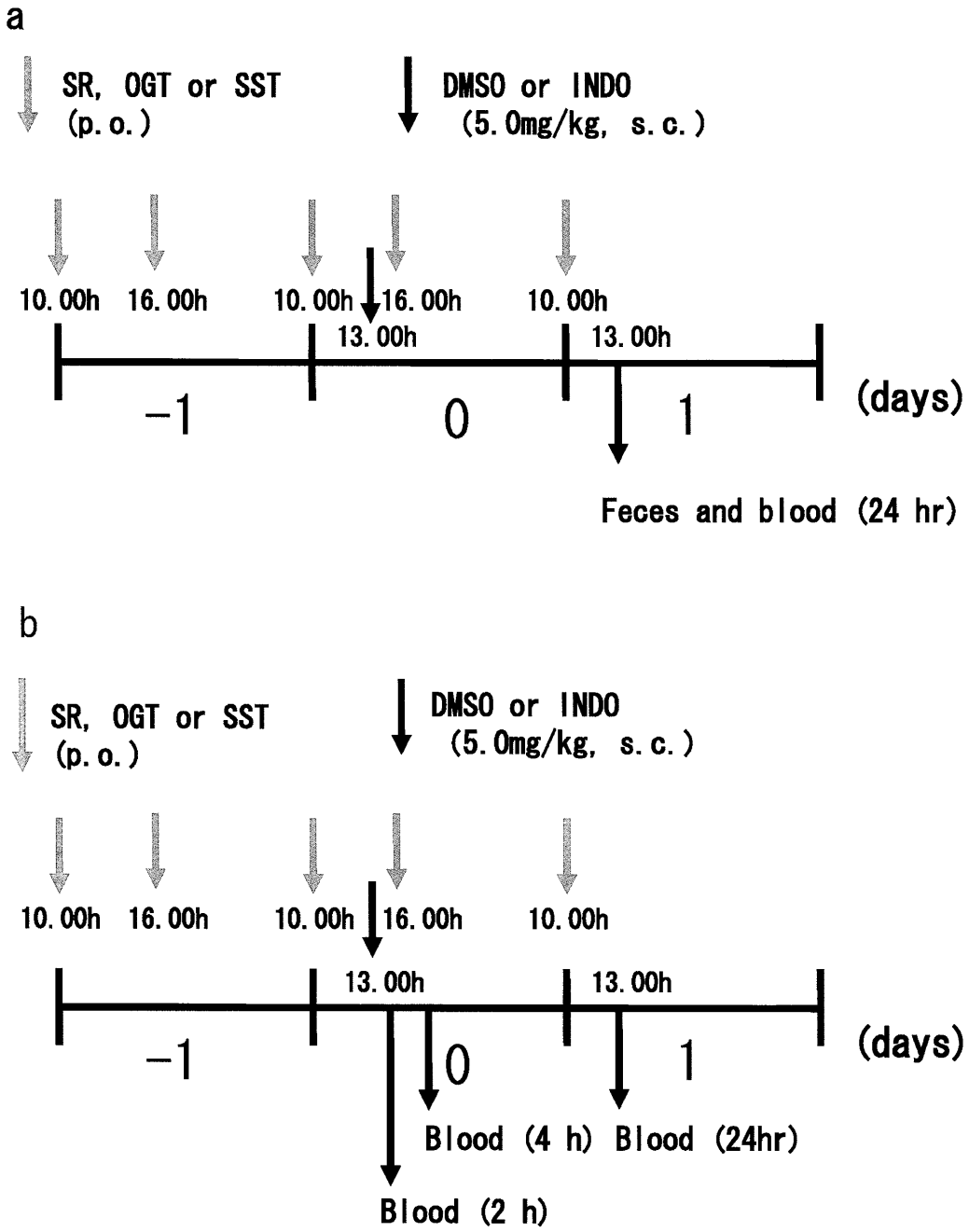
Table 1 Herbal components of OGT and SST

Orengedokuto (OGT)	Weight ratio	**San'oshashinto (SST)**	Weight ratio
COPTIDIS RHIZOMA	2	RHEI RHIZOMA	3
SCUTELLARIA RADIX	3	SCUTELLARIA RADIX	3
PHELLODENDRI CORTEX	1.5	COPTIDIS RHIZOMA	3
GARDENIAE FRCTUS	2		



Schema 1 Experimental schedules for the assessment of intestinal bleeding and blood loss (a) and plasma INDO concentration (b)

OGT and SST were suspended in distilled water and orally administered to mice twice a day at 10.00h and 16.00h. SR was administered at 250 and 500 mg/kg. OGT and SST were administered at 250, 500 and 1100 mg/kg. On day 0, INDO dissolved in DMSO was subcutaneously injected at 5 mg/kg in an injection volume of 1 μL/g body weight. The protocols for this experiment had been approved by the Committee of Animal Care and Experiments of University of Toyama.

Histological assessment of INDO-induced enteropathy. Duodenum, jejunum and ileum (~ 5 cm) were isolated from the mice treated 1 day after the treatment with vehicle or INDO and fixed in 10% (v/v) formalin. Sections prepared from the paraffin-embedded tissues were stained by hematoxylin and eosin and observed under light microscopy.

Determination of blood hemoglobin and total protein concentration. A small volume (approx. 50 μL) of blood was obtained by puncturing the retro-orbital plexus of mice with heparinized capillaries (Drumond Scientific, Broomall, PA) 24 hr after INDO administration. A portion of whole blood was used for the determination of hemoglobin (Hb) concentration by using a commercial assay kit (Hemoglobin B test Wako, Wako Pure Chem). Another portion of blood was centrifuged briefly and plasma was used to the determination of total protein concentration based on Biuret method (Biuret Reagent, Wako Pure Chem). Bovine serum albumin was used as a calibration standard for plasma total protein concentration.

Determination of fecal Hb content. Fecal Hb content was determined as described by Welch and Young.[16] The feces excreted for 24 hr after INDO administration were homogenized in distilled water using a Polytron homogenizer (Kinematica, Switzerland) at room temperature. Volume of the homogenates was adjusted to 50 ml with distilled water and a 0.5-ml aliquot of homogenates was dispensed into a glass tube closed with a screw cap and then heated at 100 ℃ for 10 min to release heme from Hb. After the tubes were cooled, 3.0 ml of acetic acid/water (30/70, v/v) was added to the homogenates and then mixed for 2 min. Ethyl acetate (2.0 ml) was added and then voltexed for 2 min. The organic upper layer containing heme was used for the determination as follows. Heme content in the upper phase was determined based on its peroxidase-like activity to decompose hydrogen peroxide in the presence of tetramethylbenzidine (TMBZ). The increase in absorbance at 660 nm for 1 min was measured at room temperature. The fecal Hb content was determined by comparing absorbance change of standard mixture containing a known amount of bovine Hb (Wako Pure Chem).

HPLC for the determination of baicalin content in SR and kampo formulations. SR, OGT or SST (0.1 g each) was suspended in 6 ml of mobile phase of HPLC described below and sonicated for 30 min. The mixture was centrifuged at 3000 rpm for 15 min. The supernatant was filtered through a membrane filter (0.45 m) and diluted 100-fold with the mobile phase of HPLC. Twenty L from the extract was used for HPLC[17] using a reverse phase C18 column (4.6X250 mm) (Senshupak, Tokyo, Japan) equilibrated with 20 %(v/v) acetonitrile in water containing 0.95 g/L ammonium tetrapentylbromide (Wako Pure Chem) at 35 ℃. Ultraviolet absorbance at 280 nm was used for the detection of baicalin. The elution time and the content of baicalin were determined by using a known amount of authentic standard (Wako Pure Chem).

Determination of plasma INDO concentration. Mice were divided into groups composed of 6 mice/group and each group of mice was administered with distilled water, SR (500 mg/kg), OGT (1100 mg/kg) or SST (1100 mg/kg) as described in Schema 1. INDO dissolved in DMSO was subcutaneously injected and a small volume of blood was obtained 2, 4 and 24 hr after INDO injection. Plasma was obtained by centrifugation and used for the determination of INDO concentration by HPLC as described previously.[18]

Statistical analysis. Statistical analysis for the data was performed by analysis of variance (ANOVA) with the Bonferroni's post-hoc test by using Prism version 4 (GraphPad Software, Inc., San Diego, CA).

Results

A single subcutaneous injection of INDO induced ulceration exhibiting mucosal detachment and external fibrin deposition in the extensive areas of duodenum and jejunum (arrows are indicating in Fig. 1). Such histological abnormalities were not shown in stomach (data not shown). On the other hand, it was found that fecal Hb content was markedly elevated in INDO-treated mice compared with the untreated mice (Fig. 2), which was accompanied by the decrease in blood Hb and total protein concentration. Thus, intestinal bleeding and blood loss were induced during INDO-induced enteropathy in mice.

Prior to INDO treatment mice were administered with SR extract and then intestinal bleeding and blood loss were compared with the control mice. It was found that fecal Hb content was significantly higher in the mice treated with a higher dose of SR extract (500 mg/kg) than in the control mice (Fig. 2). However, the decrease in blood Hb and total protein concentrations in INDO-treated mice was not significantly changed by the administration of SR extract. Since the enhancement of fecal Hb excretion in INDO-treated mice by SR extract may be due to its modification of INDO metabolism. When plasma INDO concentration was determined up to 24 hr after INDO administration (Fig. 3), no difference was shown in the change in plasma INDO concentration between the control and SR groups (500 mg/kg).

The next study was carried out to test whether SR-containing kampo formulas enhance intestinal bleeding in INDO-treated mice. The same doses of two SR-containing kampo formulations, Orengedokuto (OGT) and San'oshashinto (SST), as SR extract used in the above tests (250 and 500 mg/kg) did not change intestinal bleeding and blood loss in INDO-treated mice (Fig, 4). However, we here note that compounds derived from SR in OGT and SST are diluted with those derived from other herbal components of

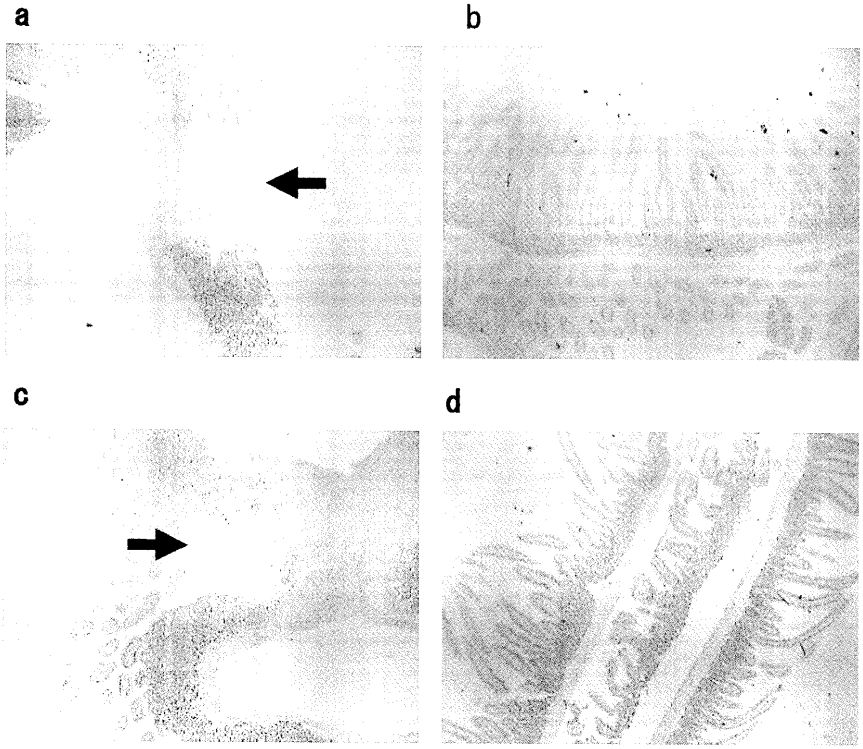


Fig. 1 Microscopic assessment of INDO-induced enteropathy
Upper (**a** and **b**) and lower (**c** and **d**) photographs indicate the sections of duodenum and jejunum, respectively. The sections **a** and **c** were prepared from INDO-treated mice and those of **b** and **d** were from vehicle-treated mice.

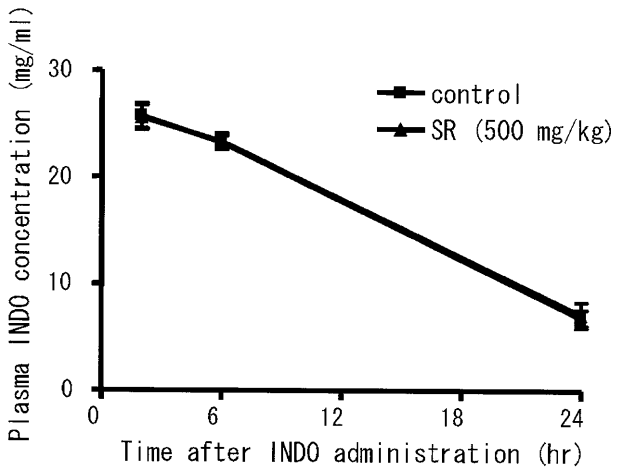


Fig. 3 Effect of SR on plasma INDO concentration
INDO was subcutaneously administered to the mice treated with or without SR extract (500 mg/kg). Blood was obtained from mice 2, 4 and 24 hr after indomethacin administration and plasma INDO concentration was determined by HPLC.

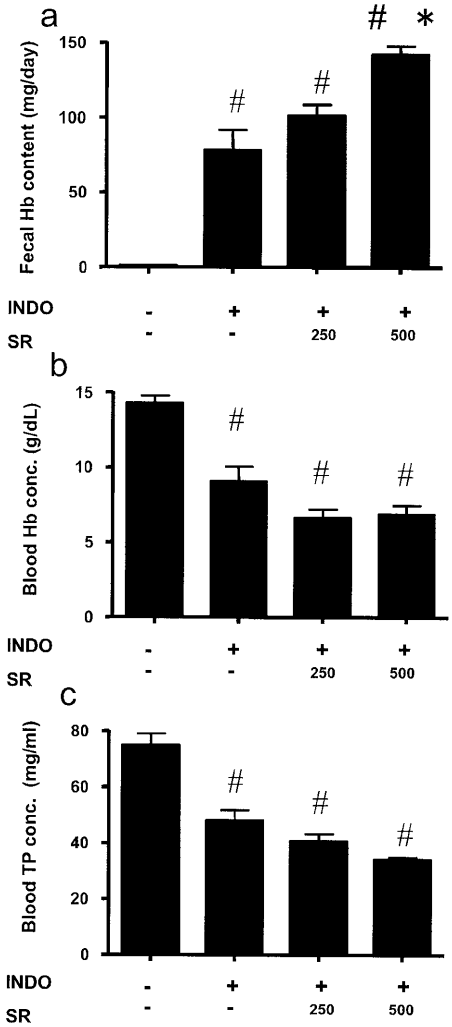


Fig. 2 Effect of oral administration of SR on intestinal bleeding and blood loss in INDO-treated mice
Mice were divided into four groups composed of 6 or 7 mice in each group. Untreated group of mice were treated with distilled water and DMSO. Control groups of mice were treated with distilled water and then INDO. Other two groups of mice were treated with oral SR (250 or 500 mg/kg) and then subcutaneous INDO. Fecal excretion of hemoglobin (Hb) (a) and blood Hb (b) and total protein concentration (c) for 24 hr after INDO treatment were evaluated. Statistically significant difference at $p<0.05$ (ANOVA with Bonferroni post hoc test) was observed between the untreated and INDO-treated groups (#) and between the control and SR-treated group (*).

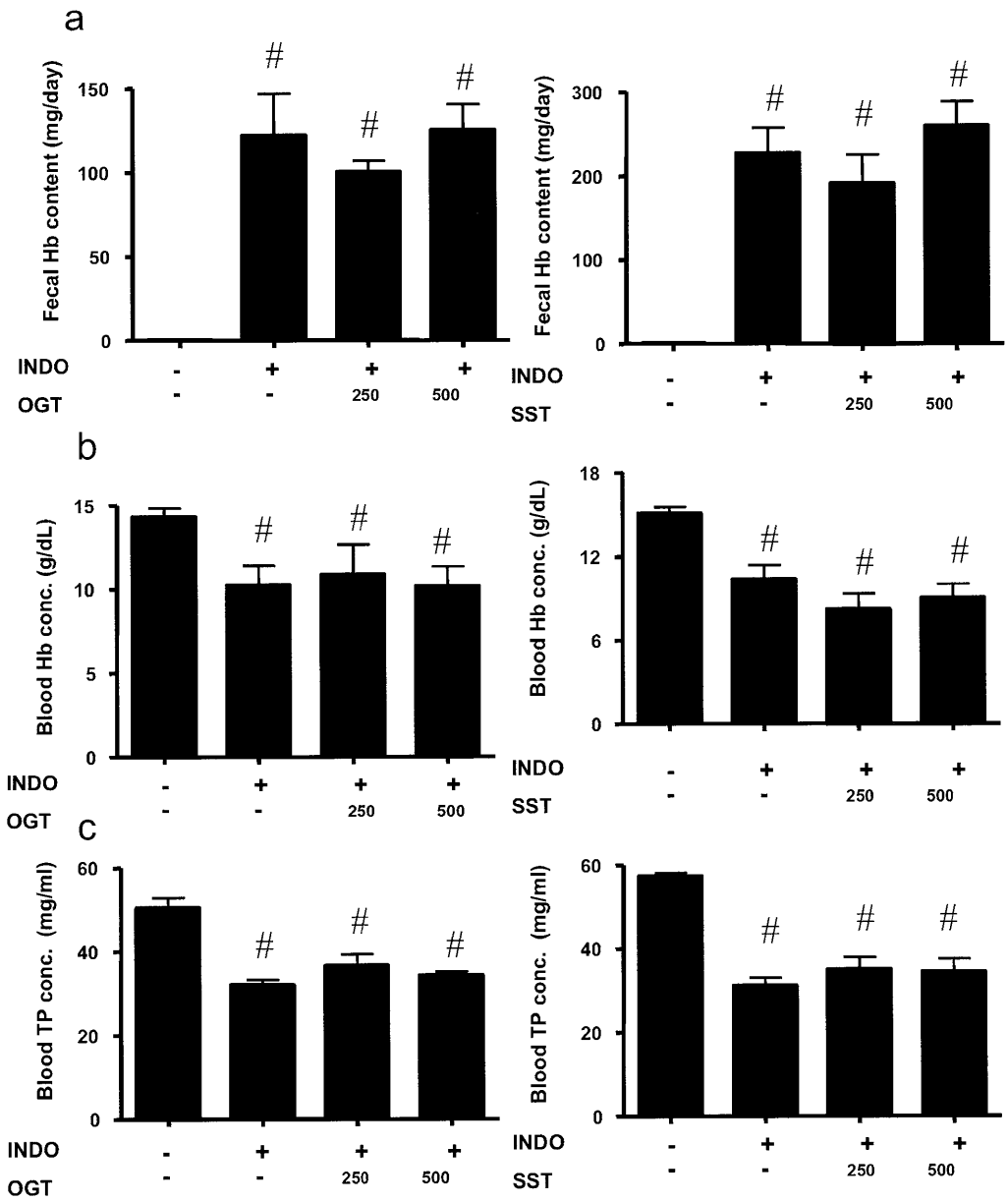


Fig. 4 Effect of oral administration of OGT and SST on intestinal bleeding and blood loss in INDO-treated mice
Mice were divided into four groups composed of 6 or 7 mice in each group. Untreated group of mice were treated with distilled water and DMSO. Control groups of mice were treated with distilled water and INDO. Other two groups of mice were treated with oral OGT (left panel) or SST (right panel) (250 or 500 mg/kg) and INDO. Fecal excretion of hemoglobin (Hb) (a)and blood Hb (b) and total protein concentration (c) for 24 hr after INDO treatment were evaluated. Statistically significant difference at $p<0.05$ (ANOVA with Bonferroni post hoc test) was observed between the untreated and INDO-treated groups (#).

these formulas. Therefore, we determined the contents of baicalin, a major compound found in SR, to estimate the overall contribution of SR-derived compounds in OGT and SST (Fig. 5). As expected, baicalin contents in OGT and SST were 14.1 and 13.6% (w/w), respectively, whereas that in SR extract was 30.1 % (w/w). Therefore, we assumed that the administration of OGT and SST at the above doses (250 and 500 mg/kg) did not enhance intestinal bleeding in INDO-treated mice as did SR extract due to lower contents of SR-derived compounds in these formulas.

A dose of OGT and SST at 1100 mg/kg, which contain similar amounts of baicalin to that in SR at 500 mg/kg, was examined for intestinal bleeding in INDO-treated mice. However, the administration of OGT and SST at this dose strongly suppressed intestinal bleeding in INDO-treated mice (Fig. 6). In addition, decreased blood Hb and total protein concentration were restored by the administration of OGT and SST. Again, it was shown that OGT and SST did not modulate the change in plasma INDO concentration (Fig. 7).

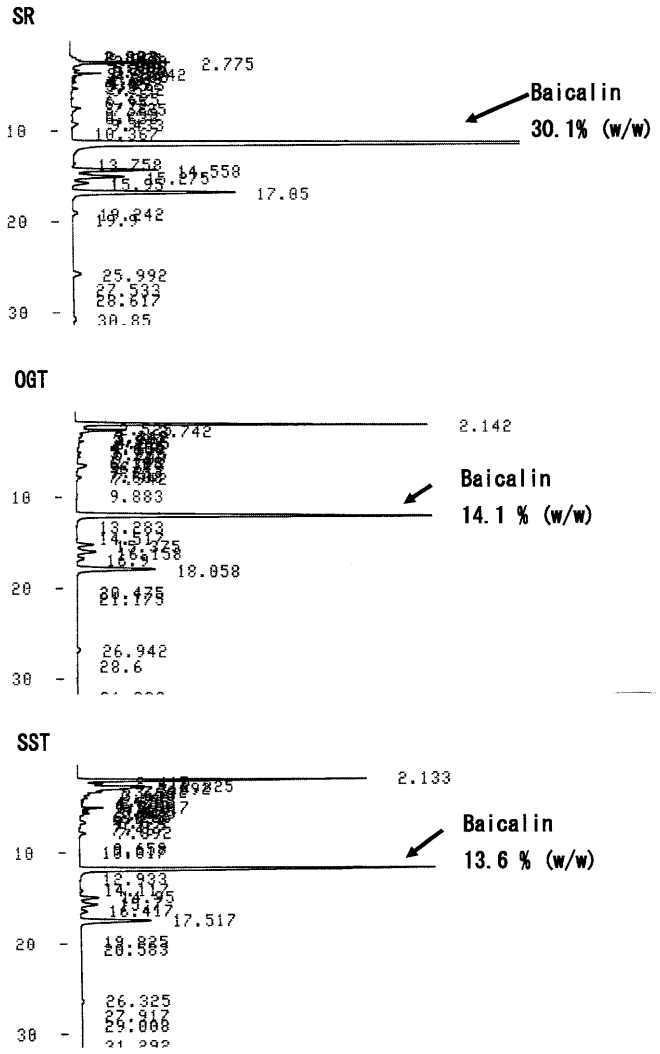


Fig. 5 HPLC determination of baicalin in SR, OGT and SST extracts
Dried extracts of SR, OGT and SST were extracted with mobile phase for HPLC and injected to HPLC. The proportions of baicalin in these extracts were shown above each chromatogram.

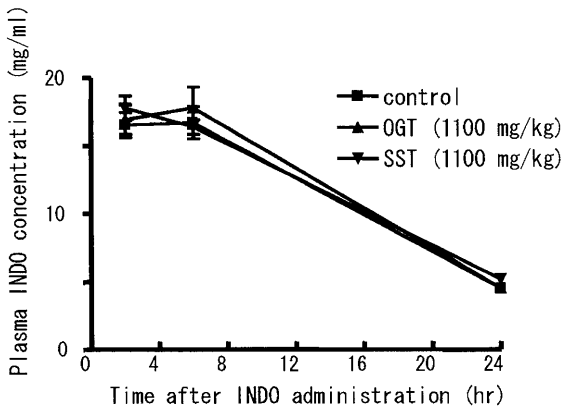


Fig. 7 Effect of OGT and SST extract on plasma INDO concentration
INDO was subcutaneously administered to the mice treated with or without OGT or SST extract (500 mg/kg). Blood was obtained from mice 2, 4 and 24 hr after indomethacin administration and plasma indomethacin concentration was determined by HPLC.

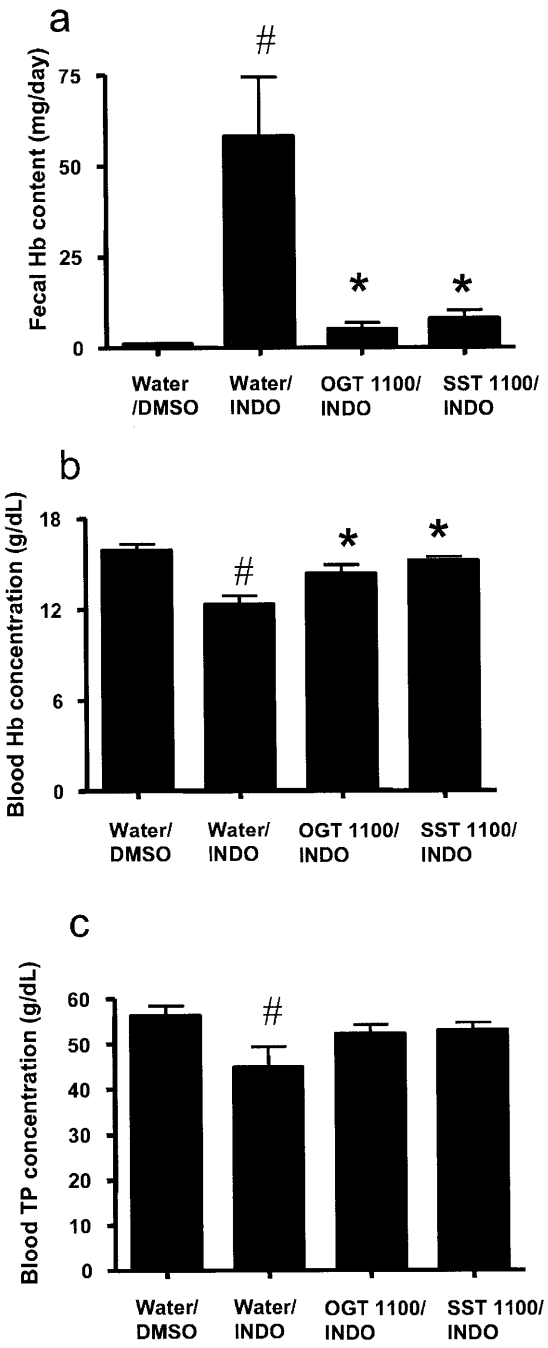


Fig. 6 Effect of oral administration of OGT and SST on intestinal bleeding and blood loss in indomethacin-treated mice
Mice were divided into four groups composed of 6 mice in each group. Untreated group of mice were treated with distilled water and DMSO. Control groups of mice were treated with distilled water and INDO. Other two groups of mice were treated with oral OGT or SST (1100 mg/kg) and INDO. Fecal excretion of hemoglobin (Hb) (a) and blood Hb (b) and total protein concentration (c) for 24 hr after INDO treatment were evaluated. Statistically significant difference at $p<0.05$ (ANOVA with Bonferroni post hoc test) was observed between the untreated and control group (#) and between the control and OGT- or SST-treated group (*).

Discussion

Baicalin is a major chemical constituent of SR extract, which accounted for 30.1 % of the SR extract (Fig. 6). It is reported that baicalin or its aglycon baicalein inhibits COX activity or suppresses COX-2 induction *in vitro*.[15,19] Therefore, baicalin could augment the inhibitory action of INDO on COX activity and subsequently enhance intestinal injury by this drug. In addition, since the inhibition of thromboxane A2 generation in platelets by INDO contributes to the enhancement of intestinal bleeding,[20] COX inhibition in the platelets by SR-derived compounds may account for the enhancement of INDO-induced intestinal bleeding. However, the roles of SR-derived compounds other than baicalin can not be excluded in the enhancement of intestinal bleeding in INDO-treated mice. The effects of purified compounds derived from SR on INDO-induced intestinal bleeding are remained to be examined.

A higher dose of OGT and SST (1100 mg/kg) which contained similar amounts of baicalin to that in SR extract administered at 500 mg/kg markedly suppressed intestinal bleeding, which was associated with the restoration of blood loss (Fig. 6). However, lower doses of OGT and SST (500 mg/kg) were ineffective for INDO-induced intestinal bleeding (Figs. 4). It is possible that a dose between 500 and 1100 mg/kg of OGT and SST might exert an intermediate suppression on INDO-induced intestinal bleeding. Further studies are necessary to examine the effects of narrow dose ranges of OGT and SST on INDO-induced intestinal bleeding. However, it is allow us to assume that the herbal constituents of OGT and SST strongly suppress the enhancing effect of INDO-induced intestinal bleeding by SR extract. Coptidis Rhizoma is a common herbal constituent in the OGT and SST (Table 1) and should be considered as the active constituents for the suppression of INDO-induced intestinal bleeding. It has been reported that Coptidis Rhizoma or its major chemical constituent such as berberine has diverse pharmacological actions under various experimental conditions,[21-23] which may explain the prevention of INDO-induced intestinal bleeding by OGT or SST. Phellodendri Cortex is also a berberine-containing herbal constituent of OGT and may contribute to the effects of this formula. In addition, reported gastrointestinal and anti-inflammatory effects of Gardeniae Fructus[24,25] and Rhei Rhizoma[26] could support the effectiveness of OGT and SST on INDO-induced intestinal bleeding. Thus, the precise roles of each herbal constituent of OGT and SST are remained to be confirmed. However, we should note that the present study represents cases that pharmacological actions of kampo formulas can not be predicted from that of a single herbal component.

To elucidate the mechanism for the modulation of INDO-induced intestinal bleeding by, OGT or SST, we examined the effect of SR, OGT and SST on the change in plasma INDO. Since no difference in the change in plasma INDO concentration among the groups, SR, OGT or SST differentially modulated INDO-induced intestinal bleeding without changing circulating levels of INDO. However, INDO undergoes enterohepatic re-circulation; INDO is conjugated with glucuronic acid in the liver and the conjugate is secreted with bile, followed by re-absorption after deconjugation by the action of gut-flora.[27] The enterohepatic re-circulation of INDO is recognized as one of the mechanisms accounting for extensive damage to the intestinal mucosa by NSAIDs.[28] Since baicalin also undergoes re-enterohepatic circulation in a similar manner to INDO,[29] luminal metabolism of INDO might be modulated by SR-derived compounds. It is also possible that compounds derived from OGT and SST might modulate luminal metabolism of INDO. Therefore, luminal metabolism of INDO should be investigated to elucidate the differential effects of SR, OGT and SST on intestinal bleeding associated with INDO-induced enteropathy.

Very recently, Miura et al have reported preventive effects of OGT on enteropathy as well as lethal toxicity in the mice treated with relatively a high dose of INDO (30 mg/kg).[30] In this study, OGT was added into the diet at 2 % (w/w) and this dose was equivalent to a dose of 2,800 mg/kg/day, which was not much different from that in our study (1,100 X 2 =2,200 mg/kg/day). Therefore, the observations by Miura et al.[30] and in the present study seem to represent a similar nature of the effect of OGT on INDO-induced enteropathy and associated intestinal bleeding. In addition, they reported the elevation of COX-2 levels in intestinal tissues by OGT administration and suggested that the restoration of reduced prostaglandin generation by INDO is implicated to the prevention of INDO-induced enteropathy by OGT. However, there are many reports demonstrating that NSAIDs initiate intestinal injury mainly through their topical effects on intestinal mucosa independent of prostaglandins.[31-33] Therefore, in addition to the role of prostaglandins the modulation of prostaglandin-independent actions of INDO should be considered in the prevention of INDO-induced enteropathy by OGT. On the other hand, since OGT and SST are used to expect hemostatic effects, the preventive effects of these formulas on INDO-induced intestinal bleeding may be due to the augmentation of blood coagulation.

Many kinds of kampo formulas are extensively used in the patients with various types of inflammatory diseases. Therefore, there are cases in which the kampo formulas are used in combination with NSAIDs during therapy for inflammatory diseases. As shown by a recent paper[28] and the present study, OGT and SST may be useful for limiting complications associated with INDO-induced enteropathy. On the other hand, since SR is contained in other kampo formulas, their influences on INDO-induced enteropathy also remain to be investigated.

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Japanese abstract

インドメタシン（INDO）の単投与により，糞中へのヘモグロビン（Hb）排泄と血中のヘモグロビンと総タンパク濃度の低下を伴う腸疾患症状がマウスにおいて誘発された。マウスにあらかじめオウゴンエキス（SR, 500 mg/kg, 2回/日）

を投与しところ，糞中へのHb排泄量が有意に増加したが，血中のHbおよび総タンパク濃度には変化しなかった。これに対して，SRを含有する黄連解毒湯（OGT）および三黄瀉心湯（SST）を1100 mg/kgにて投与したところ，INDOによる糞中へのHbの排泄や血中Hbおよび総タンパク濃度の低下は顕著に抑制された。SR, OGTおよびSSTの投与は，血中のINDO濃度の変化には影響しなかった。本研究の結果は，INDO誘発性腸疾患に伴う消化管出血や血液損失の制御において，OGTやSSTが有用である可能性を示唆する。しかしながら，これら以外のSR含有漢方方剤のINDO誘発性腸疾患に対する有害性の可能性についても検討されるべきである。

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