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Therapeutic Effect of Whole Bear Bile and Its Components against Croton Oil-Induced Rectal Inflammation in Rats

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Abstract − Bear bile has been used as a therapeutic for cerebral and coronary thrombosis, convulsion, hepatitis, jaundice, and abscess in traditional oriental medicine. In recent decades, the effects of bile acids on cancer, cholestasis, and liver injury have been investigated in many studies. In this study, we investigated the anti-inflammatory effects of whole bear bile (WBB) and its two major components, chenodeoxycholic acid (CDCA) and ursodeoxycholic acid (UDCA), on rectal inflammation in rats. Bile acids in WBB were quantitatively analyzed by HPLC. Rectal inflammation was induced in male Sprague-Dawley rats by insertion of croton oil-saturated cotton tips. WBB, UDCA or CDCA solution was orally administered to rats one hour after induction of rectal inflammation. Rats were sacrificed 4 or 24 hours after induction of rectal inflammation. The evaluation included measurement of weight and thickness of rectum and histopathologic examination of rectal tissue. Furthermore, we examined the inhibitory effect of WBB, UDCA or CDCA against NO production in LPS-stimulated RAW 264.7 cells. The contents of UDCA and CDCA in WBB were 39.26 μg/mg and 47.11 μg/mg, respectively. WBB treatment significantly reduced the weight and thickness of rectum compared with UDCA or CDCA treatment. The inhibition of NO production by WBB, UDCA and CDCA in LPS-stimulated RAW 264.7 cells was much higher than that by the control. And, WBB treatment suppressed the induction of NO synthase in rectum homogenates. These results suggest that the anti-inflammatory effect of WBB is related to the suppression of NO synthase induction and the inhibition of NO production by UDCA, CDCA and other bile acids of WBB.

Keywords: Bear bile, UDCA, CDCA, Inflammation, NO, iNOS

INTRODUCTION

Whole bear bile (WBB) was often prescribed to patients with cerebral and coronary thrombosis, convulsion, hepatitis, jaundice, and abscess by practitioners of traditional Chinese medicine. Bear bile contains an appreciable fraction of ursodeoxichlic acid (UDCA) and chenodeoxycholic acid (CDCA) (Solá *et al*., 2006). UDCA has been shown to reduce serum liver enzymes in chronic liver diseases (Poupon *et al*., 1994) and is an effective treatment for primary biliary cirrhosis and other cholestatic liver diseases (Beuers *et al*., 1998). Recently, several studies reported that UDCA had a therapeutic effect, not only on intra-hepatic diseases, but also on inflammation in other parts of the body (Podda *et al*., 1990; Fried *et al*., 1992; Earnest *et al*., 1994).

Generally, many kinds of rectal injuries such as anal fissure, anal abscess, anal fistula and perianal hematoma are called hemorrhoids. The symptoms of hemorrhoid are swelling and inflammation of veins in the rectum and anus. Various factors including constipation, chronic diarrhea, hypertension, obesity, pregnancy and excessive consumption of alcohol can cause hemorrhoids (Milsom, 1992; Loder *et al*., 1994).

Non-surgical treatment for hemorrhoids is a cream or topical analgesic containing steroids. However, creams or suppositories containing steroid preparations have several adverse effects. Steroids weaken the skin and may contribute to further flare-ups. This has led to increasing demand for natural products with anti-inflammatory activity and fewer side effects.

WBB was used traditionally against various inflammatory conditions. However, pharmacological effects and

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mechanism of the WBB remains to be not. Accordingly, we investigated the anti-inflammatory effect of bile acids on croton oil-induced rectal inflammation in rat. We focused on UDCA and CDCA as known major components of whole bear bile. As results, oral administration of WBB significantly decreased rectum weight and thickness compared with the positive control. Furthermore, its therapeutic effects were much greater than those of ibuprofen, which was used as a reference drug. WBB was also found to inhibit NO production and COX-2 expression in LPS-stimulated macrophages RAW 264.7. Further, UDCA and CDCA, components of WBB, inhibited LPS-induced NO production and COX-2 expression. These results could provide a mechanism for pharmacological activity of the bile acids, in addition to anti-inflammatory potential of WBB.

MATERIALS AND METHODS

Materials

Bear bile was purchased from Hukryounggang Province (China) and authenticated by Dr. Hwang, a botany professor at the College of Pharmacy, Chungbuk National University (Cheongju, Korea). Na-CDCA, Cholic acid (CA), Lithocholic acid (LCA) ρ-Bromophenacylbromide, croton oil, ibuprofen and Lipopolysaccharide were purchased from Sigma (St. Louis, MO, USA); RPMI medium 1640 from Gibco (Grand Island, NY, USA); Na-UDCA from Calbiochem (Cambridge, MA, USA); and Preparation H® was purchased from Ildong Pharm. Co. (Kyungkido, Korea).

Test animals

The guidelines for Animal Experimentation approved by the Laboratory Animal Research Center, Chungbuk National University were followed in these experiments. Six-week-old Sprague-Dawley albino male rats (Samtako, Kyungkido, Korea), weighing 250 ± 10 g, were used in this study. The animals were maintained under controlled conditions of temperature (23 ± 1°C), relative humidity (55 ± 10 %), and 12 hrs lighting per day. Standard laboratory chow and tap water were provided ad libitum. The rats were allowed three to four days to acclimatize to the experimental environment for three to four days before the experiments.

Derivatization of bile acids

WBB, UDCA and CDCA were derivatized with ρ-bromophenacylbromide because the reaction of bile acids with ρ-bromophenacylbromide yields quantitatively, strongly absorbing ρ-bromophenacyl esters that can be determined by HPLC with absorbance measured at 260 nm (Mingrone and Greco, 1980). Before derivatization, each bile acid was dissolved in methanol and ρ-bromophenacylbromide (1%) or triethylamine (1%) dissolved in acetonitrile. 1-ml samples were mixed with 0.5 ml each of ρ-bromophenacylbromide and triethylamine. The samples were then incubated at 80°C in a dry thermo bath for 1 hour.

Table I. Gradient condition of mobile phase used to analyze bile acids

Time (min)	Mobile phase A	Mobile phase B	Flow rate
0	30	70	0.7
4	30	70	0.7
25	0	100	0.7
30	0	100	0.7
35	30	70	0.7
45	30	70	0.7

Quantitative analysis of UDCA and CDCA in WBB by HPLC

The bile acids were quantitatively analyzed using an HPLC system (Hitachi L-6000 pump, L-6200 intelligent pump, L-4200 UV-VIS Detector and D-2500 Chromato-integrator) with a Kromasil C18 column (250×4.6 mm). The mobile phase consisted of phase A (H_2O) and phase B (methanol) with 0.2% H_3PO_4. Elution from the column was achieved with the following linear gradient (Table I).

Induction of rectal inflammation in rats

Croton oil solution was prepared before induction of rectal inflammation and kept in an ice bath. Purified water and diethylether were mixed with pyridine. The solution was then intensively stirred with diethylether containing 3% croton oil. The ratio of purified water: diethylether: pyridine: diethylether containing 3% croton oil was 1:5:4:10. Rectal inflammation was induced by insertion of 100 μl croton oil-saturated cotton tips for ten seconds by the method of Okumura.

Evaluation of rectal inflammation

Male rats were anesthetized with diethylether at 4 hrs or 24 hrs after croton oil treatment and 20 mm-length rectums were detached for analysis of inflammation. The rectal weight was measured on a balance and the thickness was measured with a precision micrometer (Mitutoyo Co, Japan). The results were expressed as the difference in weight and thickness between drug-treated rat and positive control.

Administration of UDCA, CDCA or WBB

Rats, weighing 250 ± 10 g, were assigned randomly to weight-matched groups (6 rats/group). Sodium UDCA, CDCA or WBB was dissolved in purified water and administered (each 50 mg/kg) orally one hour after induction of rectal inflammation (n=6). The amount of sodium UDCA, CDCA or WBB administered was determined based on a preliminary study. The volume administered was 2 ml/kg of body weight. Orally treated rats were sacrificed 4 hrs or 24 hrs after induction of inflammation. Ibuprofen (50 mg/kg) was used as a reference drug.

Rectum histology

Rectums were fixed in neutral formalin buffer, dehydrated in a graded ethanol series, cleared in xylene and embedded in paraffin wax. The sections were cut at 5 μm, stained with Harris' hematoxylin and eosin, and observed under a microscope.

Cell culture

RAW 264.7 cells were purchased from American Type Culture Collection (Manassas, VA, USA). They were grown in Dulbecco's modified Eagle's media supplemented with 10% FBS, benzylpenicillin potassium (143 U/ml) and streptomycin sulfate (100 μg/ml) at 37°C in a 5% CO_2 atmosphere.

NO assay

RAW 264.7 cells were treated with samples (UDCA, CDCA or WBB) for 2 hours, then stimulated with LPS (1 μg/ml) for 24 hours. Amounts of nitrite were measured according to the procedure of Archer (1993). In brief, aliquots (100 μl) of the culture media were reacted with 1:1 mixture (100 μl) of 1% sulfanilamide and 0.1% N-(1-naphthyl) ethylenediamine. The absorbance values were measured at 540 nm.

Cytotoxicity of WBB, CDCA and UDCA

Cytotoxicity was measured using the WST-1 method. Assays were performed according to the manufacturer's instructions (Dojindo, Kumamoto, Japan). Cells were seeded in 96-well plates at a density of 5×10^4 cells per well in culture medium for 24 hrs to allow attachment. The next day, the culture medium was replaced with 100 μl of serum-free medium containing 0-200 μg/ml WBB, CDCA or UDCA. The cell proliferation reagent WST-1 [2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfo phenyl-2H-tetrazolium] (20 μl) was added to each sample at 22 hrs after WBB, CDCA or UDCA treatment, and cells were incubated for an additional 2 hrs. The absorbance of the samples was measured using a Microplate reader (Molecular Devices, USA).

Western blot

Rectal tissues were homogenized and suspended in modified RIPA buffer (20 mM Tris, pH 7.4, 100 mM NaCl, 1% sodium deoxycholate, 1% Nonidet P-40, and 1% SDS) containing protease inhibitors (1 mM PMSF, 1 mM NaF, 1 mM Na_3VO_4, 1 μg/ml leupeptin, and 1 μg/ml aprotinin). Lysates were collected and subjected to western blot analysis. Briefly, equal amounts of protein were resolved on SDS-acrylamide gel by electrophoresis. The proteins were transferred to PVDF membrane and, after blocking with Tris-buffered saline containing 5% non-fat milk, the membranes were probed with anti-iNOS (Upstate) or GAPDH (Santa Cruz) and visualized by enhanced chemiluminescence reagent (GE Healthcare, Buckinghamshire, UK).

Reverse transcription-polymerase chain reaction (RT-PCR)

Macrophages RAW 264.7 were pretreated with WBB for 2 hrs and stimulated with LPS (1 μg/ml) for 5 hrs at 37°C incubator with 5% CO_2. Total RNA was extracted with TRIzol reagent according to the manufacturer's recommendations (Invitrogen; Carlsbad, CA, USA). Reverse transcription were performed using an 100 pmole oligo (dT_{18}) primer and 1 μg total RNA for cDNA synthesis. PCR analyses were performed on aliquots of the cDNA preparations to detect iNOS, cyclooxygenase (COX)-2, macrophage inflammatory protein (MIP)-1β and interferon -inducible protein (IP)-10, and β-actin (as an internal standard). The reactions were carried out in a volume 20 μl of primers (5' and 3') and Bulk AccuPower PCR PreMix (Bioneer; Alameda, CA, USA) containg 1U Taq DNA polymerase, 250 μM dNTP and reaction buffer. After an initial denaturation for 5 min at 94°C, thirty amplification cycles were performed for COX-2, MIP-1β and IP-10 (30 sec 94°C denaturation, 30 sec 58°C annealing and 1 min 72°C extension), and β-actin (30 sec 94°C denaturation, 30 sec 54°C annealing and 30 sec 72°C extension). After amplification, PCR products were electrophoresed on 1% agarose gels and visualized by ethidium bromide staining and UV irradiation.

Statistics

The data was analyzed using one-way ANOVA followed by Dunnett's test as a post hoc test in SigmaStat®. $*p<0.05$ and $**p<0.01$ were considered statistically significant.

RESULTS

Contents of UDCA and CDCA in WBB

Solá *et al*. (2006) identified the major bile acid species of black bear as CA, UDCA and CDCA by gas chromatography. In the present study, UDCA, CDCA and CA (retention time 28.95 min) were the major components of WBB (Fig. 1). The contents of UDCA and CDCA in WBB were 39.26 μg/mg and 47.11 μg/mg, respectively.

Induction of rectal inflammation

Croton oil-treated rat showed an acute inflammatory response that was characterized by congestion and increases in the weight and thickness of the rectum. The weight and thickness of rectums were checked at 1, 4, 9, 12 and 24 hours after induction of inflammation in the rectum. Inflammatory signs were increased time-dependently and reached a maximum at 12 hrs after croton oil application (Fig. 2, 3).

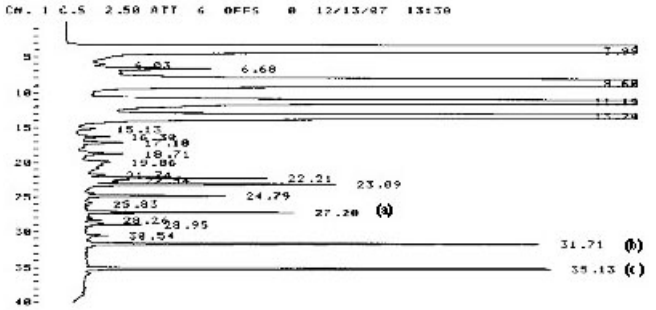

Fig. 1. HPLC chromatogram of WBB. (a) UDCA, (b) CDCA, (c) LCA (IS).

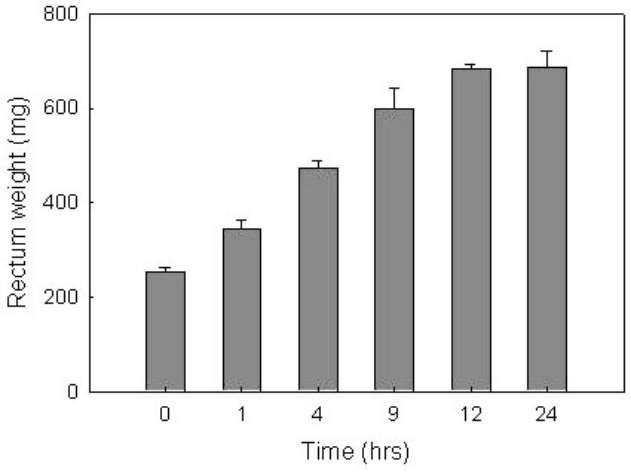

Fig. 2. The change in rectum weight. Rectum weight increased time-dependently until 12 hours and then maintained at steady state until 24 hours after croton oil treatment.

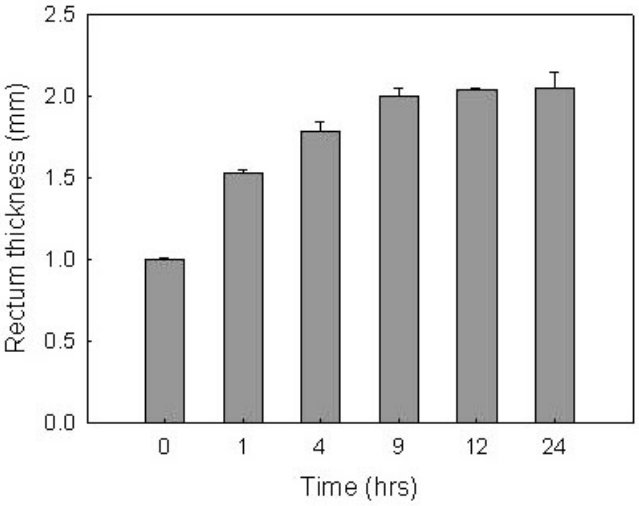

Fig. 3. The change in rectum thickness. Rectum thickness increased time-dependently until 12 hours and then maintained at steady state until 24 hours after croton oil treatment.

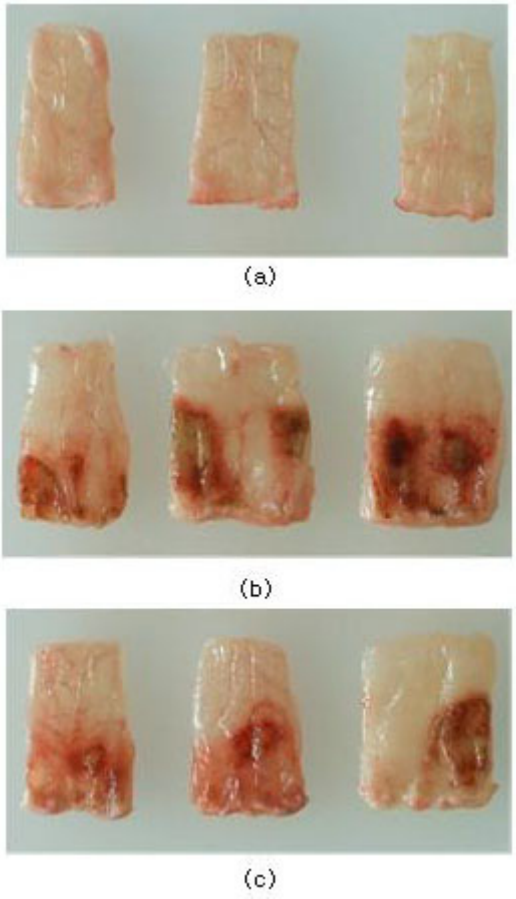

Fig. 4. Photographs of rat rectum (20 mm). (a) normal, (b) croton oil-treated, (c) WBB administered after croton oil treatment.

Photographs of rectum

In the control, the shape of the rectum was normal in appearance and the mucosal surface showed intact villi (Fig. 4A). Croton oil-treated rats had thickened and swollen rectums with severely damaged mucosal surfaces (Fig. 4B). Congestion formed as a result of direct contact with the croton oil. Meanwhile, the WBB-treated group exhibited reduced thickness of rectum compared with the positive control group, and the congestion of the mucosal surface also showed some repair (Fig. 4C). Consequently, WBB treatment effectively reduced the swelling of rectal tissue and repaired the congestion of the mucosal surface of the rectum.

Effect of orally administered WBB, UDCA and CDCA on rectal inflammation

In a preliminary study, the oral administration amount of WBB were 50, 100 and 200 mg/kg. The therapeutic effect of WBB on rectal inflammation showed dose dependent, but they didn't show significant difference (Table II). Therefore, we determined the administration amount of WBB as 50 mg/kg. As shown in Fig. 5, oral administration of WBB after croton oil treatment significantly decreased the rectal weight and thickness compared with the control group at 24 hrs after croton oil treatment. The rectal weight and thickness were 27.8% and 12.3% lower, respectively. Furthermore, its therapeutic effects were much greater than those of ibuprofen, which was used as a reference drug.

We also tested the anti-inflammatory effects of UDCA and CDCA, components of WBB, on croton oil-induced rectal inflammation. At 24 hrs after croton oil application, oral administration of UDCA and CDCA decreased the inflammatory signs in the rectum. UDCA- or CDCA-treated group showed significant differences in the weight of the rectum compared with the positive control group (Fig. 6).

Table II. Effects of oral administration of WBB on rectal weight and thickness. WBB were administered 50, 100 and 200 mg/kg, respectively

	Body weight (g)	Rectum weight (mg)	Rectum thickness (mm)
Normal	246.67 ± 2.58	187.10 ± 3.06	1.11 ± 0.04
P. Control	246.67 ± 2.58	352.80 ± 11.95	1.84 ± 0.23
WBB 50 mg/kg	256.67 ± 1.29	316.67 ± 4.21	1.62 ± 0.03
WBB 100 mg/kg	251.67 ± 1.29	309.80 ± 8.78	1.57 ± 0.12
WBB 200 mg/kg	256.67 ± 1.29	281.00 ± 7.10	1.53 ± 0.04

Data are presented as means ± SD (n=6).

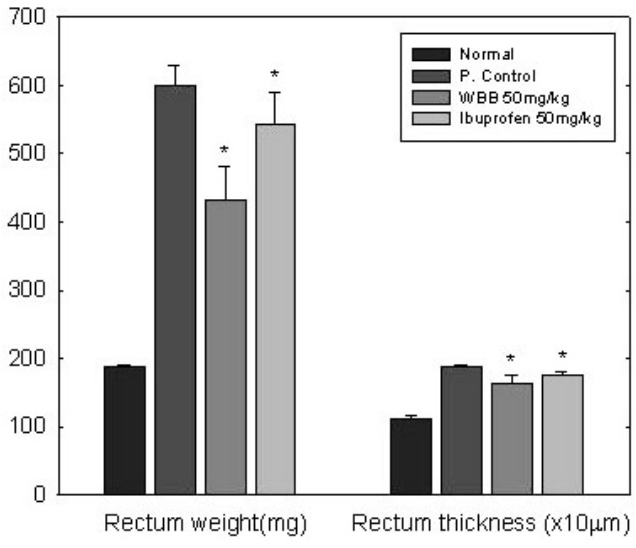

Fig. 5. Effects of oral administration of WBB on rectal inflammation. Ibuprofen was used as reference drug. Data are presented as means ± SD (n=6). * significantly different from the control value (ANOVA test, *$p < 0.05$).

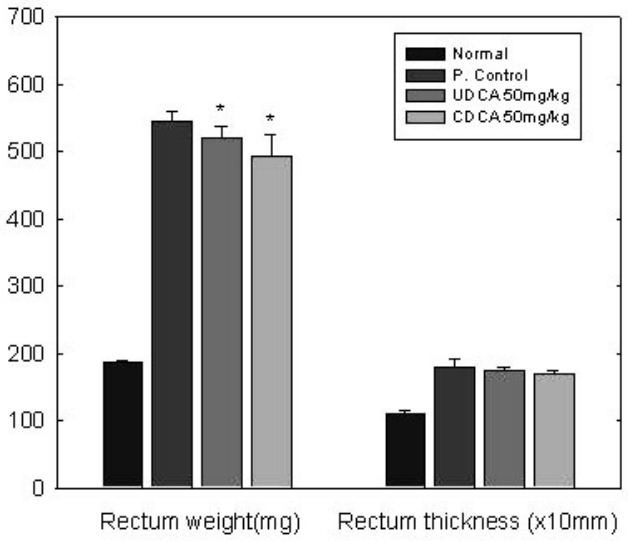

Fig. 6. Effects of oral administration of UDCA or CDCA on rectal inflammation. Data are presented as means ± SD (n=6).

Histopathology of the rectum of WBB treated rats

In the control, no histopathologic alterations were observed in the rectum (Fig. 7A). Mucosal layer and connective tissue were normal in appearance. The rectum of croton oil-treated rat showed clear signs of inflammation (Fig. 7B). They had more swollen mucosal layer and connective tissue than the normal control. Also, the connective tissue and basal layer were infiltrated with leukocytes. In the WBB-treated group, the mucosal layer showed some repair and the thickness of connective tissue and the numbers of infiltrated leukocytes were reduced compared with those of the positive control (Fig. 7C).

Inhibitory effect of WBB, UDCA and CDCA on NO production in LPS-stimulated RAW 264.7 cells

We examined the effects of WBB, UDCA and CDCA on LPS-induced NO release from RAW 264.7 cells by detecting changes in nitrite concentration in cell culture media. The cells were incubated with WBB, UDCA or CDCA for 2 hrs and then stimulated with LPS (1 μg/ml) for 24 hrs. The culture media were collected and assayed for nitrite production. As shown in Fig. 8, 9, WBB, CDCA and UDCA dose-dependently inhibited LPS-induced NO release in RAW 264.7 cells. The inhibition of NO production was CDCA (EC_{50} 12.30 μg/ml) > UDCA (EC_{50} 26.40 μg/ml) > WBB (EC_{50} 49.94 μg/ml).

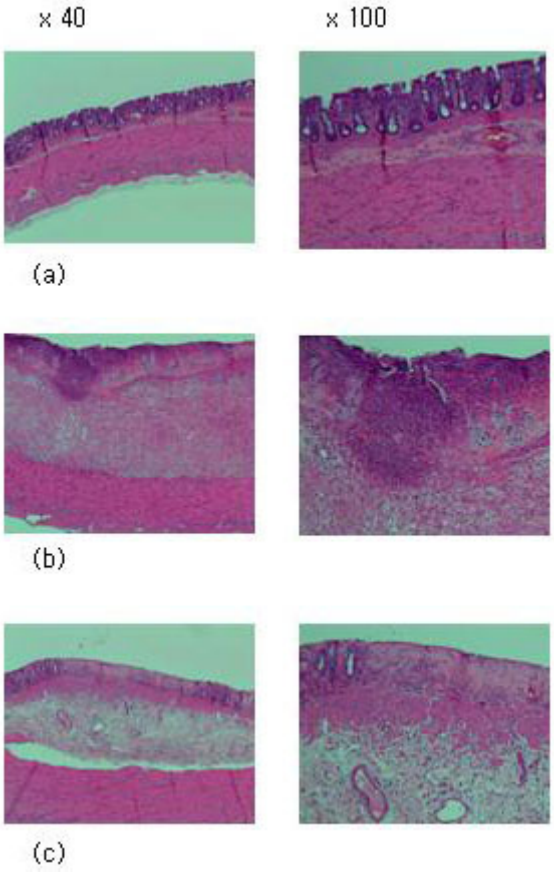

Fig. 7. Histology of rat rectum, stained with hematoxylin & eosin. Magnification, ×40 and ×200. (a) normal, (b) croton oil-treated, (c) WBB administered after croton oil treatment.

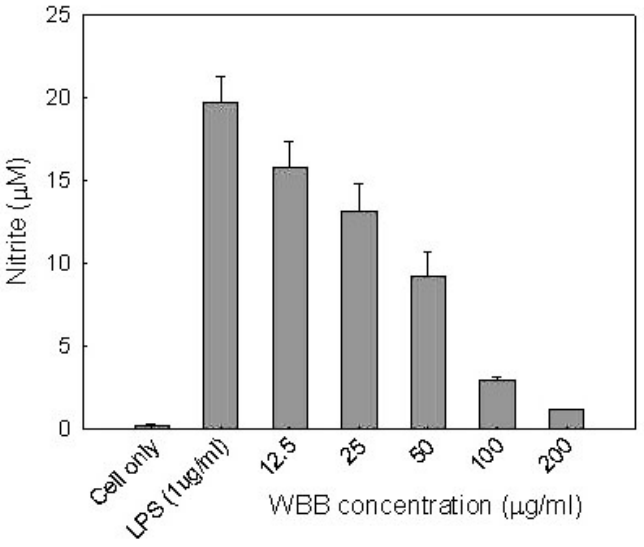

Fig. 8. The effect of WBB on LPS-induced NO production in RAW 264.7 cells. Cells were pretreated with different concentrations (12.5, 25, 50, 100, 200 μg/ml) of WBB for 2 hrs. LPS (1 μg/ml) was then added and cells were incubated for 24 hrs. Data are presented as means ± SD (n=3).

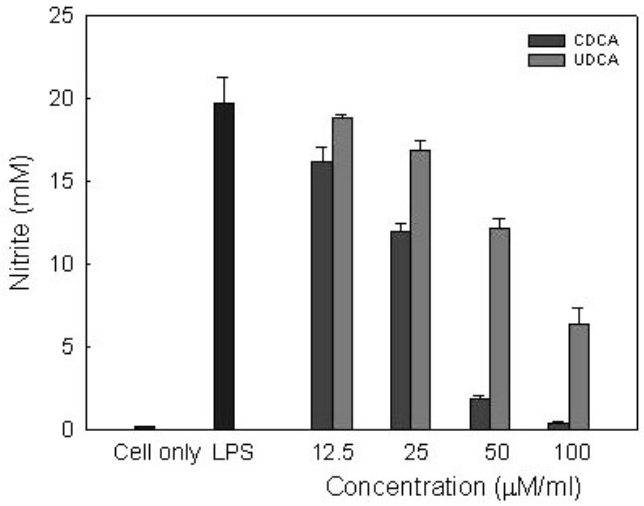

Fig. 9. Effects of UDCA and CDCA on LPS-induced NO production in RAW 264.7 cells. Cells were pretreated with different concentrations (12.5, 25, 50, 100 μM/ml) of UDCA or CDCA for 2 hrs. LPS (1 μg/ml) was then added and cells were incubated for 24 hrs. Data are presented as means ± SD (n=3).

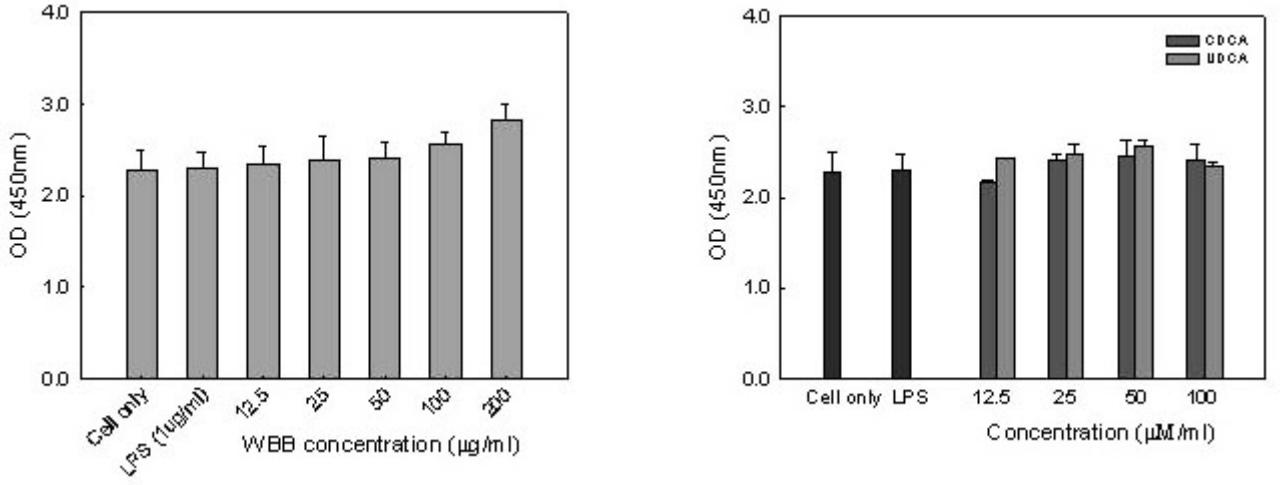

Fig. 10. Cell viability of RAW 264.7 cells treated with WBB, CDCA or UDCA for 24 hrs. Data are presented as mean ± S.D. (n=3).

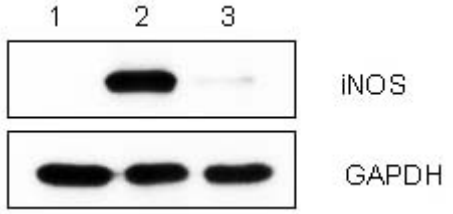

Fig. 11. The effect of WBB on croton oil-induced iNOS expression in rectum. 1. normal, 2. croton oil-treated, 3. WBB administered after croton oil treatment.

Cytotoxicity of WBB, CDCA and UDCA in RAW 264.7 cells

Treatment with various concentrations of WBB, CDCA or UDCA did not inhibit RAW 264.7 cell growth (Fig. 10). At higher concentrations of WBB (200 μg/ml), the relative number of cells increased at 24 hrs. No cell growth inhibition was observed with CDCA and UDCA.

Inhibitory effect of WBB on croton oil-induced iNOS production in rectum

Western blotting was performed to determine whether the inhibitory effect of WBB on the pro-inflammatory mediator (NO) was related to modulation of iNOS expression. In normal control, iNOS was not detected; croton oil treatment upregulated iNOS and treatment with WBB inhibited this upregulation (Fig. 11). This result indicates that the inhibitory effects of WBB on croton-induced NO production are caused by iNOS suppression.

Effect of WBB on LPS-induced mRNA expression of pro-inflammatory mediators

To determine whether WBB inhibits LPS-induced mRNA expression of pro-inflammatory mediators, such as COX-2, MIP-1β and IP-10, we examined the effect of WBB using RT-PCR. As shown in Fig. 12, WBB attenuated LPS-induced mRNA expression of COX-2 and MIP-1β in dose-dependent manners, but it didn't attenuate the expression of IP-10.

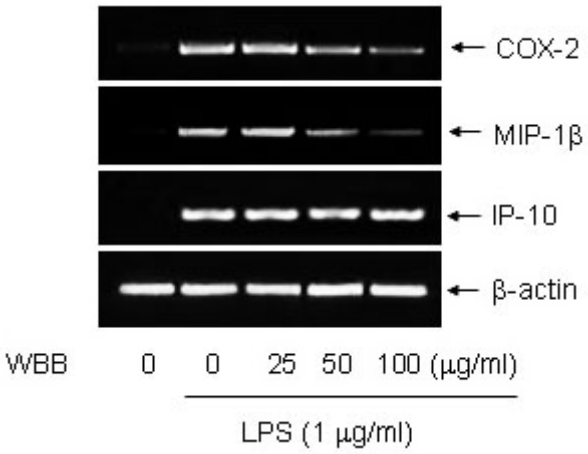

Fig. 12. Effect of WBB on LPS-induced mRNA expression of pro-inflammatory mediators. Macrophages RAW 264.7 were pre-treated with WBB for 2 hrs and stimulated with LPS (1 μg/ml) for 5 hrs. Total RNA of the cells was subjected RT-PCR.

DISCUSSION

Several mechanisms may be proposed for the anti-inflammatory effects of bile acids. Bile acids are known to induce cell damage because of their detergent properties. In general, the cytotoxic effects induced by bile acids in-

crease with increasing hydrophobicity of the bile acid species (LCA > CDCA > UDCA).

In the present study, we examined the effect of WBB and its components, UDCA and CDCA on LPS induced NO release in RAW 264.7 cells. WBB significantly inhibited NO production in LPS-stimulated RAW 264.7 cells. And, UDCA and CDCA had an inhibitory effect on NO production in LPS-stimulated RAW 264.7 cells. Based on Western blotting, iNOS was upregulated in croton oil-treated rat rectum and this upregulation was inhibited by WBB. Nitric oxide (NO) is an important inflammatory product produced by iNOS and primarily involves in promoting inflammatory response (Korhonen *et al*., 2005). Accordingly, we propose that the anti-inflammatory effect of WBB is caused by inhibition of NO production and iNOS suppression by its components, UDCA, CDCA and other bile acids. The inhibitory effect of UDCA on iNOS have previously been reported in cells (Hattori *et al*., 1996; Invernizzi *et al*., 1997), but this is the first study that WBB containing UDCA show an inhibitory effect in inflammatory induced rectum of rat.

COX-2 is inducible in the immune cells such as macrophages and synoviocytes in response to infection, injury or other stress and produces excessive amounts of PGs that serve to sensitize nociceptors and induce inflammatory states (Needleman and Isakson, 1997; Seybold *et al*., 2003). In this study, WBB was inhibited COX-2 expression in LPS-stimulated macrophages RAW 264.7. Further, UDCA and CDCA, components of WBB, inhibited LPS-induced COX-2 expression (data not shown).

We also tested the cytotoxicity of WBB, CDCA and UDCA on RAW 264.7 cells. CDCA had more potent inhibitory effects on nitric oxide production in RAW 264.7 cells, compared with UDCA. However, none of them showed any cytotoxic effects in RAW 264.7 cells by the WST-1 method. Thus, it is unlikely that bile acids inhibit iNOS via cytotoxic effects. Katsuyama *et al*. (1999) reported that the induction of iNOS appears to be dependent on the activation of nuclear factor κB, and steroid hormones such as cortisol inhibit the activation of iNOS via nuclear factor κB. Recently, UDCA was demonstrated to activate the glucocorticoid receptor in a human hepatoma cell line (Tanaka *et al*., 1996; Weitzel *et al*., 2005). Thus, similar mechanisms may be involved in the effects of bile acids on iNOS, but further studies are needed to clarify the basic mechanisms underlying the effects of bile acids on iNOS in rectal mucosa.

Conclusionally, oral administration of WBB significantly decreased rectum weight and thickness compared with the positive control. Furthermore, its therapeutic effects were much greater than those of ibuprofen, which was used as a reference drug. Accordingly, bile acid, such as UDCA and CDCA, may serve as alternatives to steroids and NSAIDs in inflammatory diseases. The anti-inflammatory effects of combined bile acids (WBB) were more greater than those of UDCA or CDCA.

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