Effects of the Geijibokryunghwan on Carrageenan-induced Inflammation and COX-2 in Hepatoma Cells

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In oriental medicine, Geijibokryunghwan(GBH) was used to improvement various symptoms created by the thrombosis. We investigated the effects of an oriental medicinal prescriptions, Geijibokryunghwan (GBH) consisting of herbs of *Cinnamomi Ramulus* (Geiji; 桂枝), *Poria cocos* (Bokryung; 茯苓), *Moutan Cortex Radicis* (Modanpi; 牧丹皮), *Paeoniae Radix* (Jakyak; 芍藥) and *Persicae Semen* (Doin; 桃仁) on tumor growth-inhibitory activity and cancer chemopreventive activity in assays representing three major stages of carcinogenesis. Cancer chemopreventive agents include nonsteroidal anti-inflammatory drugs (NSAIDS) such as indomethacin, aspirin, piroxicam, and sulindac, all of which inhibit cyclooxygenase (COX). Effects of the GBH extracts on carrageenan-induced edema inflammation using female (C57BL/6XC3H) F1 (B6C3F1) mice and tumorigenesis were examined. Finally, cyclooxygenase metabolites were determined after extracts treatment. These data suggest that GBH extracts merits investigation as a potential cancer chemopreventive agent in humans.

Key words : Chemoprevention, inflammation, COX-2, Geijibokryunghwan(GBH), thrombosis

Introduction

Cancer is common cause of death in both men and women, claiming over 6 millionlives each year worldwide. Chemoprevention, the prevention of cancer by ingestion of chemical agents that reduce the risk of carcinogenesis, is one of the ways to reduce morbidity and mortality. Cancer chemopreventive agents include nonsteroidal anti-inflammatory drugs (NSAIDS) such as indomethacin, aspirin, piroxicam, and sulindac, all of which inhibit cyclooxygenase (COX)[1-3].

This inhibitory activity is relevant to cancer chemoprevention because COX catalyzes the conversion of arachidonic acid to pro-inflammatory substances such as prostaglandins, which can stimulate tumor cell growth and suppress immune surveillance[4,5]. In addition, COX can activate carcinogens to forms that damage genetic materials[6,7].

In oriental medicine, Geijibokryunghwan(GBH) was used to improvement various symptoms created by the thrombosis. GBH formula has as formula recorded in the <Synopsis of the golden chamber>, action of 'eliminating the evil and not impairment of healthy energy' and 'promoting the flow of QI and cold and heat', so used for the expel blood stasis from the ancient[8-10].

In a previous paper, GBH was extracted with water and it was confirmed that the water GBH showed anti-thrombosis activity[11]. Thus the present paper shows the GBH extract can be used as a potential cancer chemopreventive agent in human. The effect of GBH on carrageenan- induced edema inflammation in female (C57BL/6XC3H) F1 (B6C3F1) mice and production of cyclooxygenase metabolites was also studied.

Materials and Methods

1. Cells and Materials

Female (C57BL/6XC3H) F1 (B6C3F1) mice maintained in Korea Research Institute of Bioscience and Biotechnology, KIST (Taejon, Korea) were used in all experiments. The 25-35g mice were used as the source. HepG2 cell and Hep3B cell lines were from KIST Cell bank (Taejon, Korea). Sheep red blood cells (sRBCs) were obtained from Korea Media Co., Ltd. (Seoul, Korea). RPMI 1640 were purchased from Gibco BRL (Grand Island, NY, USA). Lipopolysaccharide (LPS) and carrageenan were purchased from Sigma. GBH was obtained from Dongguk oriental hospital, Kyungju, Korea(Scheme 1).

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Scheme 1. Composition of GBH(桂枝茯苓丸)[12]

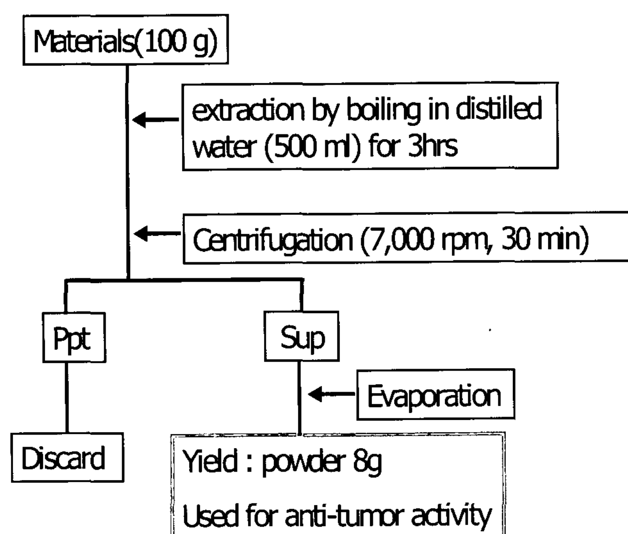
Cinnamomi Ramulus (桂枝)	1.33 g
Poria Cocos (茯苓)	1.33 g
Moutan Cortex Radicis (牧丹皮)	1.33 g
Paeoniae Radix (芍藥)	1.33 g
Persicae Semen (桃仁)	1.33 g
總　量	6.65 g

2. General analytical methods

Protein was determined by the procedure of Lowry et al.[13] using bovine serum albumin (BSA) as a standard.

3. Extraction, fractionation and purification

The dried samples were homogenized using a mechanical disintegrator with Tekmar Tissue homogenizer (Tekmar Co., Cincinati, OH, USA) in distilled water, and the crude fraction was collected by centrifugation (7,000 rpm, for 20 min) at 4℃. The supernatant solution was concentrated to about 120 ml, and used for the experiments (Scheme 2)



Scheme 2. Preparation of the water-extracts

4. Measurements of the inhibition of tumor promotion by the extract on carrageenan-induced inflammation in mice.

Female (C57BL/6XC3H) F1 (B6C3F1) mice (25-35g) were divided into groups of seven animals each. All mice received 0.1 ml of Freund's complete adjuvant (Difco; Sigma) by intradermal injection into the tail[14]. Animals were used 7 days after injection of adjuvant. One hour after oral administration of the extracts (20 or 50 mg/30 gram of body weight) or reference drugs including phenylbutazone (3 mg/30 g) and indomethacin (200 μg/30 g), the mice were injected with 0.1ml of 2% (w/v) suspension of carrageenan in saline solution into the left hind paw. For the control group, a 1:1 mixture of Tween 80 and water (0.2/3.3, v/v) and 1% (w/v) methylcellulose were used as a vehicle. The left hind paw volume of each mouse was measured by water plethysmography (Letica, Model L17500) before the adjuvant injection and, again, 6 days later, before the injection of carrageenan. Paw volumes were determined within 0 to 240 hrs after injection of carrageenan. Inhibition of edema was calculated relative to the mean edema of the vehicle-treated control group.

5. Detection of cyclooxygenase metabolites

Cyclooxygenase (prostaglandin endoperoxide synthase, E.C. 1.14.991, COX) converts arachidonic acid into prostaglandin H2, and thus produced PGH2 is converted various types of prostaglandin and finally to thromboxane A2[15]. Thus, cyclooxygenase metabolites was assayed by detecting the amounts of prostaglandin E2 (PGE2) by radioimmunoassay[16] after injecting IL-1β (0.01-10 ng/ml) in tissue.

6. Test of tumorigenesis in the two-stage mouse skin cancer model of initiation and promotion

Six groups of 20 female CD-1 mice (4 to 6 weeks old) were treated topically with 300 μmol of DMBA in 0.3 ml of acetone on the shaved dorsal region. One week later, the mice were treated with 5 μmol of TPA (12-O-tetradecanoylphorbol-13-acetate) in 0.3 ml of acetone alone or together with 5, 10, 50, 100 or 200 mg of GBH extracts, twice a week for 16 weeks. Animals were weighed weekly and observed for tumor development once every week. Then, observable skin tumors were numbered.

7. Statistical analysis

The statistical significance of difference among groups were evaluated by Student's t-test or Duncan's new multiple range test; $p<0.05$ was considered significant.

Results

1. Demonstration of the potential of the extract to inhibit tumor promotion in carrageenan-induced inflammation in mice.

We examined the anti-inflammatory activity of the extracts. In the carrageenan-induced model of inflammation in mice, the extracts significantly reduced paw edema both in the acute phase (2 to 10 hrs) and in the chronic phase (24 to 240 hrs). After female (C57BL/6XC3H) F1 (B6C3F1) mice were divided into seven groups, all mice received 0.1 ml of Freund's complte adjuvant by intradermal injection into the tail. Animals were used 7 days after injection of adjuvant. One hour after oral administration of the extratcs (20 or 50 mg/30

gram) or reference drugs including phenylbutazone (3 mg/30 g) and indomethacin (200 μg/30 g), the mice were injected with 0.1ml of a 2% (w/v) suspension of carrageenan in saline solution into the left hind paw. On the other hand, control group received a 1:1 mixture of Tween 80 and water (0.2/3.3, v/v) and 1% (w/v) methylcellulose. The left hind paw volume of each mouse was measured before the adjuvant injection and, again, 6 days later, before the injection of carrageenan. Paw volumes were determined within 3 to 240 hrs after injection of carrageenan. Inhibition of edema was calculated relative to the mean edema of the vehicle-treated control group.

The edema-suppressing activity of the extract was greater than that of phynylbutazone and was similar to that of indomethacin (Fig. 1). Overall, these results demonstrate the potential of the extract to inhibit tumor promotion.

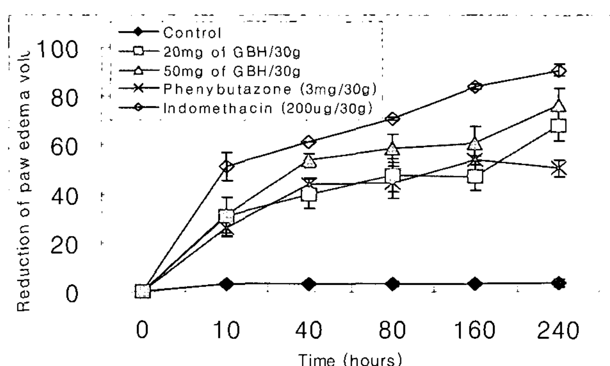


Fig. 1. Effect of the extracts (20 mg or 50 mg per 30 gram of body weight), phenylbutazone or indomethacin on carrageenan-induced inflammation in mice. Percent reduction (± S.E.) was obtained by comparing the paw edema volume in the control group (treated with carrageenan only) with that in the drug-treated group. Dosing was repeated daily for 7 days. Hours refers to hours after carrageenan injection. The data for the indomethacin group at 200 hours and 240 hrs were not reliable because of the induction of secondary lesions.

Table 1. Effect of the extracts (20 mg or 50 mg per 30 gram of body weight), phenylbutazone or indomethacin on carrageenan-induced inflammation in mice

Time (Hours)	Reduction of paw edema volume (%)				
	Control	20mg/30g	50mg/30g	Phenylbutazone	Indomethacin
0	0	0	0	0	0
10	3.3±0.6	31±7.8	31.3±7.4	26.3±5.8	51.3±3.8
40	3.3±0.6	40±6.1	53.7±2.9	44±0	61±2.6
80	3±1	47.7±6.7	58.7±5.9	44.7±0.6	70.7±6.7
160	3.3±0.6	47.3±6.1	60.7±7.0	53.7±1.2	83.7±2.1
240	3.6±1.5	68±6.2	76.3±6.7	50.7±2.3	90.7±3.5

Percent reduction (± S.E.) was obtained by comparing the paw edema volume in the control group (treated with carrageenan only) with that in the drug-treated group. Dosing was repeated daily for 7 days. Hours refers to hours after carrageenan injection. The data for the indomethacin group at 200 hours and 240 hrs were not reliable because of the induction of secondary lesions.

2. Effects of the extracts on tumorigenesis in the two-stage mouse skin cancer model of initiation and promotion

We studied tumorigenesis in the two-stage mouse skin cancer model in which DMBA was used as initiator and TPA (12-O-tetradecanoylphorbol- 13-acetate) as promoter. During an 18-week study mice treated with DMBA-plus TPA developed an average of 3.5 tumors per mouse with 34% tumor incidence. Application of 5, 10, 50, 100, 200 mg of GBH extracts together with TPA twice a week for 16 weeks reduced the number of skin tumors per mouse by 35, 43, 50, 55%, respectively, and the percentage of mice with tumors was lowered by 45, 55, 60, or 65%, respectively. No overt signs of the extracts-induced toxicity were observed, as judged by visual inspection of the skin, gross morphological examination of major organ systems, or change in body weights, relative to controls.

Table 2. Effect of GBH extracts on tumorigenesis in the two-stage mouse skin cancer model

Dose (mg) \ Weeks	Number of tumors																
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
TPA alone	0	0	0	0	0	2	3	5	12	13	21	24	25	29	33	35	36
5	0	0	0	0	0	2	4	7	11	11	19	22	24	23	31	34	35
10	0	0	0	0	0	1	3	5	11	11	16	19	23	23	27	26	29
50	0	0	0	0	0	1	1	5	7	8	14	15	19	21	26	25	28
100	0	0	0	0	0	0	2	4	5	7	13	14	16	17	20	23	24
200	0	0	0	0	0	0	2	3	4	8	12	12	13	17	18	19	19

3. Effects of the extracts and tyrosine kinase inhibitors on the accumulation of COX metabolites (COX-2 activity) in Hep3B and HepG2 cancer cells

When induction of COX was examined by treatment of tyrosine kinase inhibitors (erbstation, tyrphostin AG126) in Hep3B and HepG2 cancer cells. The accumulation of COX metabolites (PGE2) was measured from the cancer cells after 24 hrs of tumor growth. Results showed that (1) COX-2 protein is not expressed in normal cells and only low concentration of PGE2 is produced (2.9 ng/ml for 24 hrs), (2) expression of COX-2 protein is increased in cancer cells and expression of PGE2 is increased 84.5 ± 4.4 ng/ml and 47 ± 5.5 ng/ml for 24 hrs in Hep3B and HepG2, respectively (Table 3). As shown in Table 3, PGE2 production was decreased in dose-dependent manner in cancer cells.

Table 3. Effects of the GBH extracts on the accumulation of COX metabolites in Hep3B and HepG2 cancer cells

GBH Dose (mg/ml)	Release of PGE2 (ng/ml)		
	Normal cells	Hep3B cells	HepG2 cells
0	2.9 ± 0.6	84.5 ± 4.4	47 ± 5.5
0.1		83.8 ± 4.1	49.7 ± 2.4
1.0		81.7 ± 8.6	43.7 ± 1.5
5.0		78 ± 4.6	41.7 ± 6.8
50		35.1 ± 9.5*	23.2 ± 0.1*
100		21.1 ± 1.4*	24.6 ± 0.6*

The accumulation of COX metabolites (PGE2) was measured from the cancer cells after 24 hrs of tumor growth. The increase of COX activity at 24 hrs was measured by the formation of exogenous arachidonic acid (29 μM; 16 min). Data are expressed as mean ± S.E. mean from 3 determinations from at least 5 separate experimental days. * P<0.05 when compared to untreated cells at 24 hrs(C).

On the other hand, COX-2 activity on cancer cells was severely inhibited by tyrosine kinase inhibitors. Accumulation of PGE2 in Hep3B (77% inhibition in 10 μM concentration) and HepG2 (26.5% inhibition) cells were inhibited in dose-dependent manner by erbstatin and tyrphostin (Hep3B; 35.3% inhibition in 10 μM, HepG2; 26.9% inhibition) respectively (Table 4, 5). This result indicated that increased expression of COX-2 is regulated by tyrosine kinase in cancer cells.

Table 4. Dose-dependent inhibition of the accumulation of COX metabolites by erbstatin in Hep3B and HepG2 cancer cells

Erbstatin Dose (μM)	Release of PGE2 (ng/ml)		
	Normal cells	Hep3B cells	HepG2 cells
0	4.1 ± 0.6	82 ± 1.3	55.1 ± 8.1
0.01		76.9 ± 4.5	59.6 ± 4.0
0.1		69.4 ± 6.2	47.3 ± 5.6
1.0		71.7 ± 6.7	47.3 ± 6.0
10.0		27 ± 3.5*	40.5 ± 4.7*

The accumulation of COX metabolites (PGE2) was measured from the cancer cells of after 24 hrs. The increase of COX activity at 24 hrs was measured by the formation exogenous arachidonic acid (29 μM; 16 min). Data are expressed as mean ± S.E. mean from 3 determinations from at least 5 separate experimental days. * P⟨0.05 when compared to untreated cells at 24 hrs(C).

Table 5. Dose-dependent inhibition of the accumulation of COX metabolites by tyrphostin in Hep3B and HepG2 cancer cells

Tyrphostin Dose (μM)	Release of PGE2 (ng/ml)		
	Normal cells	Hep3B cells	HepG2 cells
0	3.6 ± 0.8	81 ± 5.8	54.3 ± 8.1
0.01		79 ± 8.0	64.7 ± 1.5
0.1		70.5 ± 6.1	49.3 ± 5.3
1.0		64.8 ± 2.6	45.1 ± 2.9
10.0		52.4 ± 8.1*	39.7 ± 6.6*

The accumulation of COX metabolites (PGE2) was measured from the cells of after 24 hrs. The increase of COX activity in cancer cells at 24 hrs was measured by the formation of exogenous arachidonic acid (29μM; 16 min). Data are expressed as mean ± S.E. mean from 3 determinations from at least 5 separate experimental days. * P⟨0.05 when compared to untreated cells at 24 hrs(C).

Discussion and Conclusion

On the basic of the results, in the carrageenan-induced model of inflammation in mice, the extracts significantly reduced paw edema both in the acute phase and in the chronic phase, as inhibition of edema was calculated relative to the mean edema of the vehicle-treated control group. The edema-suppressing activity of the extract was greater than that of phenylbutazone and was similar to that of indomethacin, demonstrating the potential of the extract to inhibit tumor promotion.

The effects of GBH was studied on tumorigenesis in the two-stage mouse skin cancer model using DMBA as initiator and TPA (12-O-tetradecanoylphorbol-13-acetate) as promoter, tumor generation was reduced without extracts-induced toxicity. The extracts and tyrosine kinase inhibitors inhibited the accumulation of COX metabolites (COX-2 activity) in HepG2 and Hep3B cancer cells. It has been reported that cyclooxygenase is existed as 2 forms in epithelium and induced by endotoxin, and induction by inflammatory cytokines is inhibited by tyrosine kinase[17]. COX (prostaglandin endoperoxide synthase, E.C. 1.14.991), which converts arachidonic acid to prostaglandin H2, then thromboxane A2 via prostaglandin[18]. Cyclooxygenase-1 (COX-1) is localized in endothelial cell and cyclooxygenase-2 (COX-2) is induced by pro-inflammatory stimulatory molecules such as mitogen[19], cytokine[20] and bacterial LPS[21,22]. Even though there is no any information on COX isoforms in lung, COX-2 in macrophage of human lung is inducible by LPS stimulation[23]. Cytokines can induce COX in lung epithila cells and the COX is regulated by tyrosine kinase. Therefore, induction of COX was examined by treatment of tyrosine kinase inhibitors (erbstation, tyrphostin AG126) in ovary and HepG2 cancer cells. PGE2 production was decreased in dose-dependent manner of the GBH in cancer cells. COX-2 activity on cancer cells was severely inhibited by tyrosine kinase inhibitors and slightly by GBH, indicating that increased expression of COX-2 is regulated by tyrosine in cancer cells.

We investigated the effects of an oriental medicinal prescriptions, Geijibokryunghwan (GBH) consisting of herbs of *Cinnamomi Ramulus* (Geiji; 桂枝), *Poria cocos* (Bokryung; 茯苓), *Moutan Cortex Radicis* (Modanpi; 牧丹皮), *Paeoniae Radix* (Jakyak; 芍藥) and *Persicae Semen* (Doin; 桃仁) on tumor growth-inhibitory activity and cancer chemopreventive activity in assays representing three major stages of carcinogenesis. Effects of the extracts on carrageenan-induced edema inflammation using female (C57BL/6XC3H) F1 (B6C3F1) mice and tumorigenesis were examined. Finally, cyclooxygenase metabolites were determined after extracts treatment. These data suggest that GBH extracts merits investigation as a potential cancer chemopreventive agent in humans.

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