

Inhibition of Mast Cell-Dependent Allergy Reaction by Extract of Black Cohosh (*Cimicifuga racemosa*)

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ABSTRACT

Black cohosh (*Cimicifuga racemosa*) has been used as therapeutics for pain and inflammation in Korean folk medicine. The potential effects of black cohosh extract (BCE) on mast cell-dependent allergy reaction, however, have not been well elucidated yet. In the present study, we investigated the effect of BCE on the allergy reaction using mast cell-dependent in vivo and in vitro models. BCE showed no potential of skin sensitization in local lymph node assay (LLNA). The oral administration of BCE significantly inhibited the anti-IgE-induced passive cutaneous anaphylaxis (PCA) reaction. BCE also showed inhibitory potential on the compound 48/80-induced histamine release from rat peritoneal mast cells. In addition, BCE inhibited the IL-4, IL-5 and TNF- α mRNA induction by PMA and A23187 in human leukemia mast cells, HMC-1. These results demonstrated that BCE has an anti-allergic

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potential and it may be due to the inhibition of histamine release and cytokine gene expression in the mast cells.

Key Words: Mast cells; Allergy reaction; Black cohosh; Local lymph node assay (LLNA).

INTRODUCTION

Mast cells play a key role in allergic reactions through the release of numerous inflammatory and immuno-modulatory mediators, which is triggered by antigen-induced IgE receptor cross-linking. These include histamine, proteases, leukotrienes, prostaglandins and cytokines. As a consequence, clinical manifestations of acute allergic responses such as vasodilation, increased vascular permeability, broncho-contraction and edema, are induced.^[1] In addition, allergic responses also stimulate the influx and activation of a variety of inflammatory cells including eosinophils and lymphocytes. It is strongly believed that tissue infiltration by large numbers of eosinophils is one of consistent features in the chronic inflammatory responses.^[2] And rapidly released mediators and numerous cytokines produced by mast cells are thought to be involved in inducing and sustaining these responses. Therefore, it is regarded that the effective inhibition of immediate phase reaction is the most important step in the treatment of both acute and chronic allergic inflammation.^[3] Many investigators have concentrated their efforts on finding the effective therapeutics for allergic inflammation, using well-established mast cell-dependent experimental model systems. For instance, the passive cutaneous anaphylaxis (PCA) reaction induced by injection of anti-IgE antibody has been used successfully to test anti-allergic effects of potential drugs.^[4,5] Recently, the interest of complementary and alternative (CAM) medicine against allergic inflammation is growing. In spite of limited scientific evidences and lack of mechanistic understanding, CAM has been increasingly attractive around the world, because of traditional belief of both safety and efficacy. For example, *Acanthopanax senticosus*, *Fagopyrum esculentum* Moench and *Petasites hybridus* extract were reported as possible agents for treatment of allergic diseases.^[6–8] Black cohosh is one of the widely used medicinal plants. It has been previously documented that black cohosh gives beneficial effects on menopausal symptoms such as postmenopausal cardiovascular disease, osteoporosis, hot flash, and depression.^[9,10] Besides, black cohosh has long been used as therapeutics for pain and inflammation in Korean folk medicine. The precise action mechanism of black cohosh, however, remains to be elucidated. In the present study, we have demonstrated the anti-allergic effects of black cohosh extract (BCE) using rodent experimental models and in vitro cell culture systems.

MATERIALS AND METHODS

Materials

Black cohosh was purchased from Kyeonghui Co. (Geumsan, Chungnam, Korea). Sprague-Dawley (SD) rats and Balb/c mice were supplied from Dae-Han Biolink (Eumsung, Chungbuk, Korea). Compound 48/80, anti-DNP IgE, DNP-HSA, metrizamide,



Evans blue and 2,4-dinitrochlorobenzene (DNCB) were purchased from Sigma (St. Louis, MO, USA). Iscove's modified Dulbecco's medium (IMDM), α -minimal essential medium (α -MEM), RPMI1640 medium and fetal calf serum (FCS) came from Gibco BRL (Gaithersburg, MD, USA). [methyl- ^3H]thymidine (40–60 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). M-MLV reverse transcriptase and Taq polymerase were purchased from Promega (Madison, WI, USA).

Preparation of Black Cohosh Extract (BCE)

Black cohosh was crushed and extracted with cold methanol for 1 week. The methanol-extract was concentrated in a vacuum evaporator, and the resulting residue was weighed and dissolved in appropriate vehicles.

Local Lymph Node Assay (LLNA)

LLNA was carried out according to OECD guideline^[11] with slight modifications. Briefly, the dorsa of both ears of Balb/c mice were applied with 25 μl of the appropriate dilutions of BCE in propylene glycol for 3 days. At day 4, the animals were sacrificed and the lymph nodes were excised. Single cell suspensions were prepared by gentle mechanical desegregation through 200-mesh stainless steel gauze. Lymph node cells were washed twice with an excess of HBSS, then resuspended with RPMI1640 medium, seeded onto 96-well plate (1.0×10^5 cells/well). Cells were then received 0.4 μCi of [^3H]thymidine and further incubated for 24 hr at 37°C in an atmosphere of 5% CO_2 . Incorporation of [^3H]thymidine was measured by liquid scintillation counter. Results were expressed as the stimulation index according to OECD guideline.

Passive Cutaneous Anaphylaxis (PCA) Reaction

Male SD rats, each weighing approximately 200 g, were passively sensitized according to the method previously described.^[12] The dorsal skins of rat were injected intradermally with 0.5 μg of anti-dinitrophenyl (DNP) IgE, followed 48 h later by injection of 1 μg of DNP-HSA in PBS containing 1% Evans blue via tail vein. BCE was suspended in DW at appropriate concentrations and orally administered once a day for 3 days. Intradermal injection of DNP-HSA was performed 2 h after oral administration of BCE at day 3. After 60 min, the rats were sacrificed and reaction sites were excised for determination of extravasated dye. The amount of dye was measured as previously reported.^[13] Briefly, skin tissue was solubilized with 1.0 N KOH at 37°C overnight. Then, 9 volumes of a mixture of 0.6 N KOH and acetone (5:13) was added and Evans blue dye was extracted. The absorbance of extracted dye was measured at 620 nm in a spectrophotometer.

Histamine Release from Rat Peritoneal Mast Cells (RPMC)

Rat peritoneal mast cells (RPMC) were purified as previously described^[7] with slight modifications. Briefly, SD rats were anesthetized by ether and buffer solution (150 mM NaCl, 0.1% glucose, 3 mM NaHCO_3 , 3.7 mM KCl, 3.5 mM NaH_2PO_4 , 10 U heparin) containing 0.1% FBS was injected intraperitoneally. After gentle massage, the peritoneal cavity was opened and the fluid was collected. Mast cells were separated from major components of rat peritoneal cells using 22.5% metrizamide. Mast cells of



Table 1. Nucleotide sequence of the primers and expected size of PCR products.

Primer	Sequence	Size (bp)
IL-4	Forward (5' → 3')	TGAACAGCCTCACAGAGCAG
	Reverse (5' → 3')	CTCTGGTTGGCTTCCTTCAC
IL-5	Forward (5' → 3')	GAGACCTTGGCACTGCTTTC
	Reverse (5' → 3')	ATCTTTGGCTGCAACAAACC
TNF- α	Forward (5' → 3')	AGCCCATGTTGTAGCAAACC
	Reverse (5' → 3')	ATGTTTCGTCCTCCTCACAGG
G3PDH	Forward (5' → 3')	GAGTCAACGGATTTGGTCGT
	Reverse (5' → 3')	TTCAGCTCAGGGATGACCTT

90–95% purity were routinely obtained, as estimated by toluidine blue staining. For testing histamine release, 2×10^5 /ml of RPMC was resuspended with α -MEM, FCS (1:1) and preincubated for 20 min at 37°C. BCE was dissolved in DMSO and added 30 min before treatment of compound 48/80. After incubation with compound 48/80 (50 μ g/ml) for 20 min at 37°C, the cells were separated from the released histamine by centrifugation at $400 \times g$ for 5 min. Residual histamine was released by disrupting the cells with perchloric acid and centrifugation. The amount of histamine was determined by o-phthalaldehyde spectrofluorometric method.^[12] The fluorescence was measured at 440 nm (excitation at 360 nm).

Culture of HMC-1 Cells and RT-PCR Analysis

HMC-1 cells, which are immature human leukemic mast cells,^[14] were kept routinely in IMDM supplemented with 2 mM L-glutamine, 200 U penicillin/ml, 200 μ g streptomycin/ml and 10% FCS. For RT-PCR analysis, 1×10^6 /ml of HMC-1 cells were preincubated with serum-free medium for overnight and activated with a combination of phorbol myristate acetate (PMA, 50 nM) and calcium ionophor A23187 (250 nM) for 4 h at 37°C.^[15] BCE was dissolved in DMSO and added to the incubation medium 30 min prior to the treatment of PMA and A23187. Total RNAs were extracted by acid guanidinium thiocyanate-phenol-chloroform method. Two μ g of total RNAs were reverse transcribed with M-MLV reverse transcriptase in the presence of random hexamer. The resultant RT mixtures were then subjected to PCR cycles as follows: 94°C for 30 sec, 62°C for 30 sec, 72°C for 1 min for 30 cycles (IL-4, TNF- α), 35 cycles (IL-5) and 20 cycles (G3PDH). Primers for amplifying the respective fragments are listed in Table 1. After agarose gel electrophoresis, PCR products were quantified using a densitometer.

Statistical Analysis

The results obtained were statistically evaluated using Student's t-test. Statistical significance was set at $P < 0.05$.

RESULTS

To evaluate the potential effect of BCE on skin sensitization, we carried out local lymph node assay (LLNA). As shown in Fig. 1, BCE showed lack of a significant



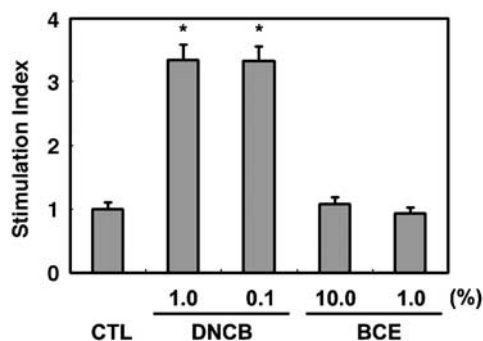


Figure 1. Effect of black cohosh extract (BCE) on skin sensitization. The dorsa of both ears of Balb/c mice were applied with BCE for 3 days, and then lymph node cells were prepared. The proliferating potential of lymph node cells incubated in vitro was measured by [^3H]thymidine incorporation. Data are expressed as stimulation index according to OECD guideline. The mean values $\pm$ SE are averages of triplicate measurements (* $P < 0.05$ vs. CTL).

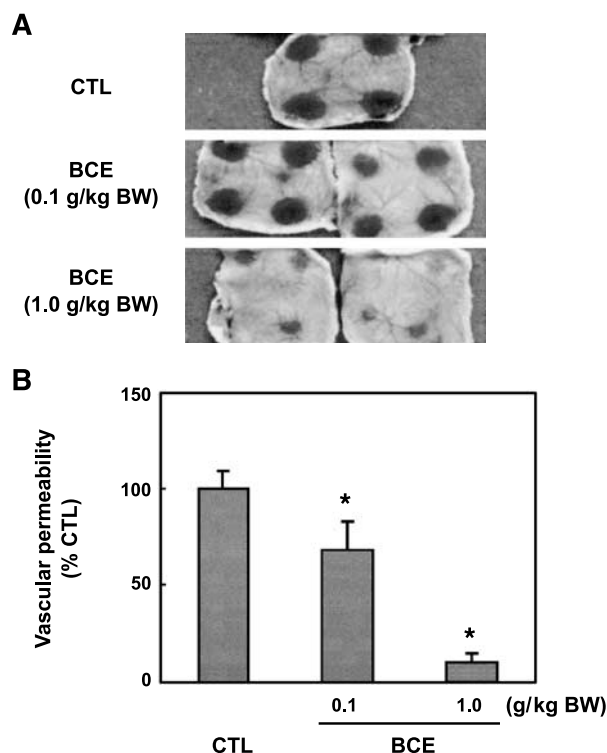


Figure 2. Effect of black cohosh extract (BCE) on passive cutaneous anaphylaxis (PCA) reaction. BCE was orally administered for 3 days at indicated concentrations, and PCA reaction was induced by injection of anti-DNP IgE and DNP-HSA. (A) Representative data of PCA reaction documented by Evans blue extravasation. (B) Quantification of effect of BCE on PCA reaction. Data are expressed as percentage of control (CTL). The mean values $\pm$ SE are averages of triplicate measurements (* $P < 0.05$ vs. CTL). (View this art in color at www.dekker.com.)



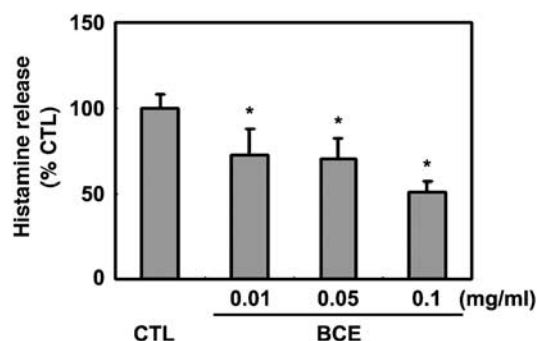


Figure 3. Effect of black cohosh extract (BCE) on compound 48/80-induced histamine release from rat peritoneal mast cells (RPMC). RPMC were preincubated with BCE for 30 min prior to incubation with compound 48/80. The amounts of histamine release from RPMC were expressed as percentage of control (CTL). The mean values $\pm$ SE are averages of triplicate measurements (* $P < 0.05$ vs. CTL).

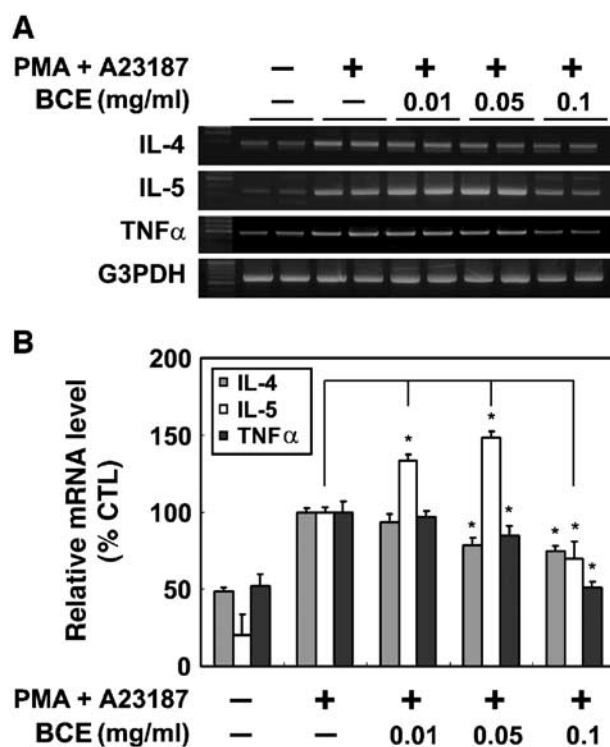


Figure 4. (A) Effect of black cohosh extract (BCE) on the mRNA level of cytokines in human leukemia mast cells, HMC-1, by semi-quantitative RT-PCR analysis. HMC-1 cells were pretreated with BCE at indicated concentrations and then activated by PMA and calcium ionophor A23187. (B) Quantification of RT-PCR products for cytokines using a densitometer. The amount of RT-PCR products for each cytokines was corrected according to the quantity of G3PDH and expressed as a percentage of control (mean $\pm$ SE, * $P < 0.05$).



potential to cause skin sensitization, while DNCB, a positive control, provoked strong skin sensitization reaction. We then examined the effect of BCE on passive cutaneous anaphylaxis (PCA) reaction. It has been well documented that PCA reaction is a typical model for mast cell-dependent immediate hypersensitivity and can be used to assess anti-allergic activities of potential drugs.^[4,5] When orally administered for 3 days, BCE produced marked inhibitory effect (93% and 32% inhibition at the doses of 1.0 and 0.1 g/kg bodyweight) on PCA reaction induced by anti-DNP IgE and DNP-HSA (Fig. 2). Previous evidences indicated that PCA reaction is tightly related with the degranulation of mast cells.^[16] We therefore tested the effect of BCE on compound 48/80-induced histamine release from rat peritoneal mast cells (RPMC). As shown in Fig. 3, BCE clearly inhibited compound 48/80-induced histamine release from RPMC, which showed 49%, 31% and 29% inhibition at the doses of 0.1, 0.05 and 0.01 mg/ml respectively. It has been well known that mast cell activation lead to not only the degranulation of numerous mediators, but also the up-regulation of many cytokine genes.^[17] In order to explore the effect of BCE on the mRNA levels of several cytokines implicated in pathogenesis of allergic inflammation, RT-PCR analysis was carried out using human leukemia mast cells, HMC-1 (Fig. 4A). When activated with PMA and ionophor A23187, the mRNA levels of IL-4, IL-5 and TNF- α were increased in HMC-1 cells. Treatment of BCE resulted in dose-dependent inhibition of IL-4 and TNF- α mRNA induction by PMA and A23187. For IL-5, BCE increased slightly the mRNA level in low concentrations, however, decreased significantly in high dose treatment (Fig. 4B).

DISCUSSION

In the present study, we have demonstrated that BCE has potent inhibitory effects on anti-DNP IgE-induced PCA reaction and compound 48/80-induced histamine release from RPMC. In addition, BCE showed prominent inhibitory potential on the gene expression of IL-4, IL-5 and TNF- α induced by PMA and A23187 in human leukemia mast cells, HMC-1. Mast cells exert their effector functions by releasing numerous mediators and cytokines. Among those, histamine is still regarded as a principal mediator of antigen-induced skin reactions.^[1] Therefore, inhibition of the production and secretion of histamine from the mast cells is likely to provide the most important tool for treatment of allergic responses. This notion is well supported by the clinical efficacy of several anti-histamines such as H₁-blocker azelastine, loratadine and cetirizine.^[3] Besides, it has been well known that human mast cells are a source of several multifunctional cytokines that play important roles in establishing chronic allergic inflammation.^[18] For instance, IL-4 released from the mast cells was implicated in the pathogenesis of allergic inflammation such as asthma and rhinitis.^[19,20] It is thought that IL-4 may enhance the selective eosinophil recruitment through induction of the endothelial expression of VCAM-1, thereby contributes to establishing chronic allergic inflammation.^[21] This is supported by evidence that ear swelling in late phase reaction was significantly suppressed by the treatment with anti-IL-4 monoclonal antibody in ovalbumin-immunized mice model.^[22] In addition, previous study indicated that mast cells are a source for IL-5 production in allergic inflammatory responses.^[20] It has been well established that IL-5 exerts its critical roles in establishing chronic allergic inflammation through the selective activation and



accumulation of eosinophils, which lead to subsequent tissue damage in infiltrated sites.^[2] TNF- α is a key mediator in many cytokine-dependent inflammatory events. TNF- α is released in allergic responses from both mast cells and macrophages via IgE-dependent mechanisms. It has been well known that TNF- α induces chemotaxis of neutrophils and T-cells, enhances cytotoxicity of macrophages, and stimulates the expression of adhesion molecules from endothelial cells.^[23] Previous data also showed that TNF- α released from mast cells is responsible for eosinophil survival through partial inhibition of apoptosis, thereby contributes to chronic inflammation.^[24] In this regard, our results suggest that BCE could affect the acute allergic reaction as well as chronic inflammation through the regulation of cytokines gene expression in the mast cells. Black cohosh (*Cimicifuga racemosa*) is a perennial plant that is native to North America, China and India. Recently, black cohosh has become an extremely popular alternative medicine for conditions that affect women's reproductive organs including menstrual problems, inflammation of the uterus and/or ovary, infertility, threatened miscarriage, and relief of labor pains.^[25] Although there are some contradictory data on the estrogenic potential of black cohosh, it is still used as an important material in estrogen replacement therapy.^[26] Besides, it has been reported that black cohosh has potential to scavenge reactive oxygen species (ROS), and the polyphenolic compounds of black cohosh such as caffeic acid, ferulic acid and cimicifugic acid, contribute to anti-oxidant potential.^[27] Interestingly, mast cells are known to generate intracellular ROS in response to antigen challenge, which may be involved in histamine release.^[28] Evidence indicated that naturally occurring polyphenolic anti-oxidants reduced ROS levels in antigen-IgE activated mast cells and concomitantly inhibited histamine release.^[29] Therefore, it could be suggested that the anti-allergic action of BCE is partly due to anti-oxidant potential of polyphenolic components in BCE. The precise action mechanism of BCE and its components, however, remains to be further investigated. In summary, we have demonstrated that BCE has profound anti-allergic potential in mast cell-dependent test models, suggesting that BCE may be a good candidate for the treatment of allergic inflammation.

ACKNOWLEDGMENT

This study was supported by a grant of the Oriental Medicine R&D Project, Ministry of Health and Welfare, Republic of Korea (HMP-00-0-21600-0011).

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