Effects of *Acer okamotoanum* sap on the function of polymorphonuclear neutrophilic leukocytes *in vitro* and *in vivo*

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Abstract. Sap is a plant fluid that primarily consists of water and small amounts of mineral elements, sugars, hormones and other nutrients. *Acer mono* (*A. mono*) is an endemic Korean mono maple which was recently suggested to have health benefits due to its abundant calcium and magnesium ion content. In the present study, we examined the effects of sap from *Acer okamotoanum* (*A. okamotoanum*) on the phagocytic response of mouse neutrophils *in vivo* and rat and canine neutrophils *in vitro*. We tested the regulation of phagocytic activity, oxidative burst activity (OBA) and the levels of filamentous polymeric actin (F-actin) in the absence and presence of dexamethasone (DEX) *in vitro* and *in vivo*. Our results showed that DEX primarily reduced OBA in the mouse neutrophils, and that this was reversed in the presence of the sap. By contrast, the phagocytic activity of the mouse cells was not regulated by either DEX or the sap. Rat and canine polymorphonuclear neutrophilic leukocytes (PMNs) responded *in vitro* to the sap in a similar manner by increasing OBA. However, regulation of phagocytic activity by the sap was different between the species. In canine PMNs, phagocytic activity was enhanced by the sap at a high dose, while it did not significantly modulate this activity in rat PMNs. These findings suggest that the sap of *A. okamotoanum* stimulates neutrophil activity in the mouse, rat and canine by increasing OBA *in vivo* and *in vitro*, and thus may have a potential antimicrobial effect in the PMNs of patients with infections.

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Introduction

Sap is a plant fluid carried through the xylem cells, which transport water and nutrients in the plant, or through the phloem sieve tube elements (1). Sap primarily consists of water with a small amount of mineral elements, sugars, hormones and other nutrients (1). *Acer mono* (*A. mono*) is an endemic Korean mono maple (2) whose sap may be ingested directly as a beverage or concentrated into syrup by boiling it for use as a sweetener (1,3). It has been suggested that the sap of *A. mono* may be beneficial due its abundant calcium and magnesium ion content. A solution of *A. mono* sap ameliorates osteoporosis-like symptoms, and is therefore known as 'bone-benefit water' (1).

Neutrophils, the most abundant type of white blood cells, are phagocytes that play critical roles in combating acute infection and innate immunity (4). These cells have a unique capacity to engulf and eliminate microbes and quickly congregate at the site of infection (5). The phagocytic responses (ingesting microorganisms or particles) of neutrophils against pathogens involve the polymerization and rearrangement of cellular actin filaments (6). The phagocytozed pathogens are then eliminated by microbicidal hydrolytic enzymes and an oxidative burst caused by the formation of reactive oxygen species (ROS) (5). Although increased production of phagocyte-derived ROS may damage host cells and tissues, ROS function as potent antimicrobial agents that protect against infection and cellular signaling molecules under certain conditions (7).

It has been reported that constituents extracted from *Acer okamotoanum* (*A. okamotoanum*) leaves and twigs have antioxidant activities (2). However, the effects of the sap on phagocytic responses have not been studied. The objective of the present study was to determine whether the sap of *A. okamotoanum* affects phagocytic responses of peripheral blood polymorphonuclear neutrophilic leukocytes (PMNs). For this, we performed experiments using mouse PMNs compromised by treatment with dexamethasone (DEX). DEX is a well-known glucocorticoid that impairs the innate functions of phagocytes (8,9). We also examined the *in vitro* effects of *A. okamotoanum* sap on phagocytic capacity, oxidative burst activity (OBA) and filamentous polymeric actin (F-actin) levels of mouse PMNs. Our results showed that the

sap of *A. okamotoanum* enhanced phagocytic activity and OBA in mouse and canine neutrophils, thus demonstrating its antimicrobial effect.

Materials and methods

Experimental animals and treatments. Male, 9-week-old ICR mice were obtained from KOATECH (Gyeonggi, Korea). The animals were housed in polycarbonate cages and acclimated in an environmentally controlled room (23±2˚C; relative humidity, 50±10%; frequent ventilation; 12-h light/dark cycle) prior to use. The sap of *A. okamotoanum* was prepared as previously described (1). To assess the effect of the sap on DEX-treated PMNs, the mice were randomly divided into eight groups. A total of 50 ml tap water or 25, 50 or 100% solutions of sap were administered to the mice at ages 9-16 weeks. Mice in the control group received a physiological saline (0.9% NaCl) solution and the other mice (treatment group) received a DEX solution (Sigma-Aldrich Co., St. Louis, MO, USA). Mice in the treatment group received three injections of DEX (1 mg/kg, subcutaneous) every 24 h. For the control group, an equivalent volume of saline solution was injected at the same time points. The experimental procedures were approved by the Ethics Committee of Chungbuk National University (Chungbuk, Korea).

PMN isolation. PMNs were then isolated from rat and canine peripheral blood vessels using a double density gradient centrifugation method to immediately collect blood samples, as described previously (10,11). Briefly, heparinized blood samples were overlaid on a Histopaque-1077 solution (specific gravity, 1.077; Sigma-Aldrich Co.) and Histopaque-1119 solution (specific gravity, 1.199; Sigma-Aldrich Co.) and centrifuged at 700 x g for 40 min at 20˚C. The PMNs were subsequently harvested from the interface between Histopaque-1077 and Histopaque-1119, and washed three times with cold phosphate-buffered saline (PBS). To purify the PMNs, residual erythrocytes were lysed by treatment with 0.83% NH_4Cl in a tri(hydroxymethyl)-aminomethane-based buffer (pH 7.2) for 5 min. PMN purity in the final cell suspension was verified to be >96% as determined by Wright-Giemsa staining analysis of a blood film obtained by cytocentrifugation. The viability of the PMNs, as determined by trypan blue dye exclusion, was >97% in every case. The resulting PMNs were resuspended in RPMI-1640 medium (Sigma-Aldrich Co.) supplemented with 2 mM L-glutamine, 0.02 mg gentamicin/ml and 5% heat-inactivated fetal bovine serum (Invitrogen, Grand Island, NY, USA).

Simultaneous measurement of phagocytic capacity and OBA. Phagocytic capacity and OBA were evaluated simultaneously as previously described (12). Briefly, the isolated PMNs were placed in 24-well plates at a density of $1x10^6$ cells/ml/well and incubated for 2 h at 37˚C with 5% CO_2 in a humidified atmosphere. For the *in vitro* assay, the PMN cells were isolated from rat and canine vessels (abdominal aorta and median antebrachial vein) and incubated with *A. okamotoanum* sap for 2 h with the previously indicated doses at 37˚C in a 5% CO_2-humidified atmosphere. A carboxylate-modified polystyrene fluorescent microsphere suspension (20 μl; 1.0 μm in size; TransFluoSpheres; Molecular Probes Inc., Eugene, OR, USA) adjusted to a concentration of $1x10^9$ beads/ml was added to the wells for the final 1 h of culture. When 15 min of culture time remained, 1 μM dihydrorhodamine 123 (Sigma-Aldrich Co.) was added. The conversion of non-fluorescent dihydrorhodamine 123 into fluorescent rhodamine 123 by intracellular ROS was used to measure OBA. The phagocytic capacity was determined by estimating the number of PMNs that had phagocytized fluorescent microspheres in the gated cell population of the sample.

The cultured cells were gently harvested, centrifuged at 400 x g for 3 min at 4˚C and washed three times with PBS solution containing 3 mM EDTA. All steps performed following the start of cultivation were conducted in the dark. The cells were analyzed within 30 min using a multipurpose flow cytometer (FACS Calibur system; Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) with an argon laser set at 488 nm and analysis software (CELLQuest, version 3.3; Becton Dickinson Immunocytometry Systems). The FL1 channel was set to 505-545 nm to detect green fluorescent rhodamine 123 and the FL3 channel was set to 630-660 nm to detect red fluorescent microspheres. The cells were gated on the basis of their forward and side light-scattering characteristics. Phagocytic capacity and OBA were expressed as percentages and mean fluorescence intensities (MFIs, arbitrary units), respectively.

Measurement of total cellular F-actin contents. Total cellular F-actin levels were measured as described previously (13). The isolated PMNs were seeded in 24-well plates at a density of $1x10^6$ cells/ml/well, and then incubated with an extract of *A. okamotoanum* sap for 80 min at 37˚C in a 5% CO_2-humidified atmosphere. A carboxylate-modified polystyrene fluorescent microsphere suspension (20 μl; 1.0 μm in size) adjusted to a concentration of $1x10^9$ beads/ml was added to the wells for the final 20 min of culture. The cultured cells were gently harvested, centrifuged at 400 x g for 3 min at 4˚C and washed three times with PBS containing 3 mM EDTA. The viability of the PMNs was verified to be >98% based on trypan blue dye exclusion. The cells were fixed with fixation buffer (BD Cytofix; Becton Dickinson Biosciences) at 4˚C according to the manufacturer's instructions, washed three times with PBS and then stained in the dark for 15 min at 37˚C with 165 nM FITC-labeled phalloidin (Sigma-Aldrich Co.) and 100 μg/ml lysophosphatidylcholine. The cells were washed and analyzed within 30 min using a FACS Calibur system (Becton Dickinson Immunocytometry Systems) and analysis software (CELLQuest, version 3.3; Becton Dickinson Immunocytometry Systems) with the argon laser set at 488 nm. Samples from 10,000 cells were assayed in triplicate. The FL1 channel was set to 505-530 nm to detect the green fluorescing FITC molecule. The F-actin levels were expressed as MFI.

Statistical analyses. The analyses were performed with SigmaStat, version 2.0 (SPSS Inc., Chicago, IL, USA). Differences between the treatment groups were evaluated by a one way analysis of variance (ANOVA), followed by Dunnett's post hoc test. Two-group comparisons were performed with a two-sample t-test. Normality tests (Kolmogorov-Smirnov) were performed to determine whether or not the results had a standard normal distribution. $P<0.05$ was considered to indicate a statistically significant difference. Results are shown as the mean ± standard deviation (SD).

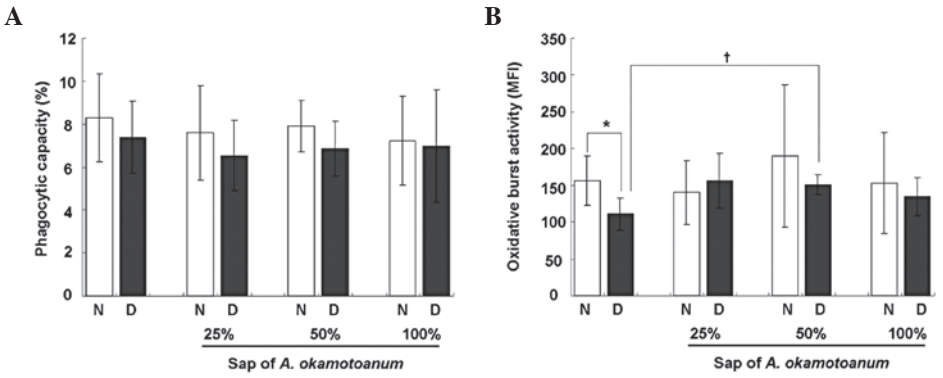

Figure 1. Effect of *Acer okamotoanum (A. okamotoanum)* sap on (A) phagocytic capacity and (B) OBA of mouse PMNs. Mice were fed *A. okamotoanum* sap (0, 25, 50 and 100%) or saline (0.9% NaCl) solution (white bars) for 6 weeks in the presence or absence of DEX (1 mg/kg) solution (black bars). Phagocytic capacity and OBA were simultaneously measured from mouse PMNs. *Significant difference (P<0.05) between the treatment groups; †Significant difference (P<0.05) vs. the control group without dexamethasone (DEX) treatment. N, no treatment; D, DEX treatment; MFI, mean fluorescence intensity; OBA, oxidative burst activity; PMNs, polymorphonuclear neutrophilic leukocytes.

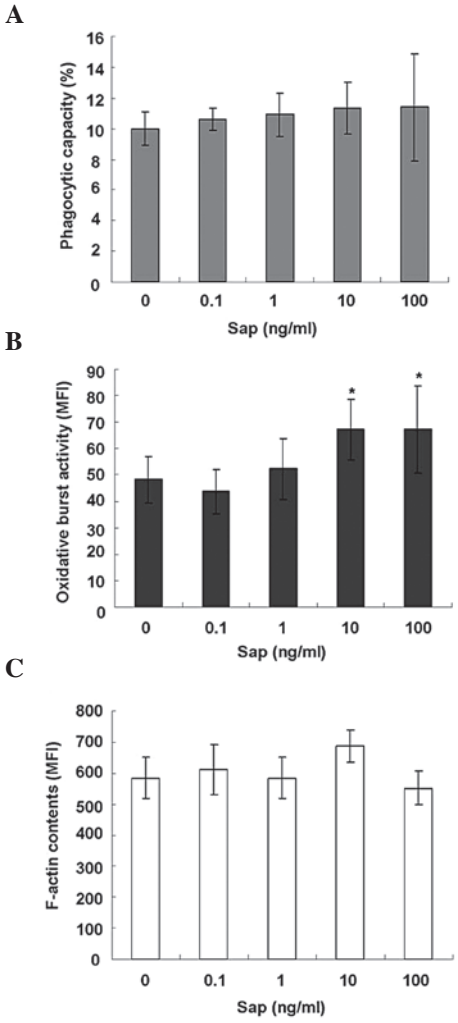

Figure 2. Effects of the *Acer okamotoanum (A. okamotoanum)* sap extract on (A) phagocytic capacity, (B) OBA and (C) F-actin content (cellular fluorescence intensity) of rat PMNs *in vitro*. PMNs were isolated from peripheral blood vessels of rats and treated with fluorescent microspheres ($2x10^7$ beads/ml/well) during the final 1 h of culturing and dihydrorhodamine 123 for the final 15 min. To measure the total cellular F-actin levels, isolated PMNs were cultured for 60 min and then incubated with fluorescent microspheres ($2x10^7$ beads/ml/well) for another 20 min. *Significantly different compared with the sap-treated groups (P<0.05). MFI, mean fluorescence intensity; OBA, oxidative burst activity; PMNs, polymorphonuclear neutrophilic leukocytes.

Results

Sap of A. okamotoanum reverses DEX-induced reduction of OBA in vivo. To investigate the effect of *A. okamotoanum* sap on phagocytic responses, mature (9-week-old) mice were fed increasing concentrations (0, 25, 50 and 100%) of *A. okamotoanum* sap for 6 weeks and were then injected with DEX (1 mg/kg) for the last 3 days. Following treatment, PMNs were isolated from the mice and the phagocytic capacity and OBA were evaluated. The OBA of the PMNs from mice that received only DEX was significantly decreased (P=0.004) compared with OBA in cells obtained from the saline-treated group (Fig. 1B). By contrast, the phagocytic capacity of the PMNs was not significantly altered by treatment with DEX (P=0.426; Fig. 1A). The reduction of OBA by DEX was reversed by pretreatment with *A. okamotoanum* sap at a concentration of 50%, indicating that the sap affected OBA *in vivo*.

Sap of A. okamotoanum enhances OBA in rat in vitro PMNs. The effects of *A. okamotoanum* sap on phagocytic capacity, OBA and F-actin content was also determined *in vitro* using rat PMNs. Similar to the results of the *in vivo* study, the *A. okamotoanum* sap extract had no significant effect on the phagocytic capacity (P=0.907; Fig. 2A). F-actin content (P=0.136; Fig. 2C) of the rat PMNs was also unaltered by this treatment. However, the OBA of the rat PMNs was increased by treatment with the extract in a dose-dependent manner. These increases were significant at concentrations of 10 and 100 ng/ml *A. okamotoanum* sap (P=0.008; Fig. 2B).

Sap of A. okamotoanum increases phagocytic activity and OBA in canine in vitro PMNs. In addition to rat PMNs, we also examined the effects of *A. okamotoanum* sap on canine PMNs to determine whether *A. okamotoanum* sap had antimicrobial activity in other species. We tested various doses (0.01, 0.1, 1, 10 and 100 ng/ml) of the sap and found that the effects on phagocytic activity (Fig. 3) were different from that of rat PMNs. The sap increased phagocytic capacity at a dose of 100 ng/ml in canine PMNs while significant changes were not observed in rat PMNs. The effect on OBA in canine

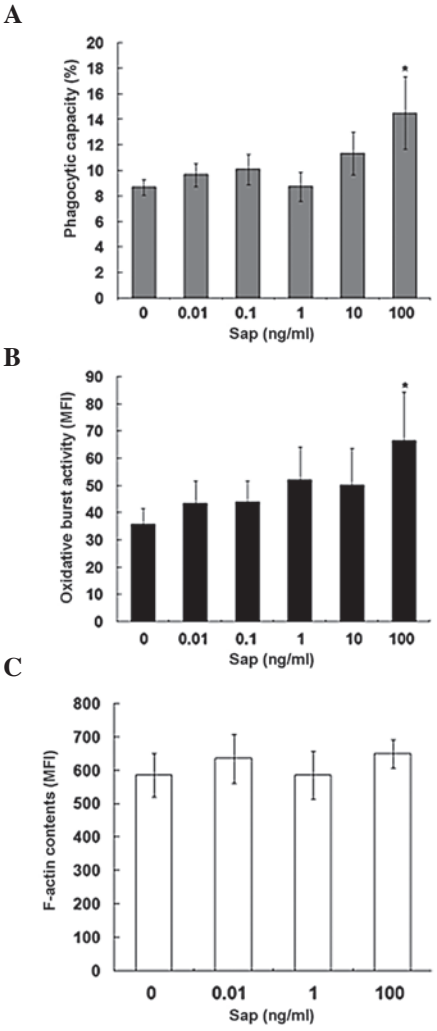

Figure 3. Effects of the *Acer okamotoanum (A. okamotoanum)* sap extract on (A) phagocytic capacity, (B) OBA and (C) F-actin content (cellular fluorescence intensity) of canine PMNs *in vitro*. PMNs were isolated from peripheral blood vessels of canines and treated with fluorescent microspheres ($2x10^7$ beads/well) for the final 1 h of culturing and dihydrorhodamine 123 for the final 15 min. To measure the total cellular F-actin levels, isolated PMNs were cultured for 60 min and then incubated with fluorescent microspheres ($2x10^7$ beads/ml/well) for another 20 min. *Significantly different from those of the sap-treated groups ($P<0.05$). MFI, mean fluorescence intensity; OBA, oxidative burst activity; PMNs, polymorphonuclear neutrophilic leukocytes.

PMNs was similar to that observed in rats; this activity was significantly augmented at a high dose of the sap (100 ng/ml). Canine PMN F-actin content, following treatment with the sap at 0.01, 1 and 100 ng/ml doses, was not significantly altered. These results were similar to those revealed in the rat PMNs (Fig. 3C).

Discussion

In the present study, we examined the effects of *A. okamotoanum* sap on the phagocytic response of canine and mouse neutrophils. First, we monitored changes in phagocytic activity, OBA and F-actin contents in the absence and presence of DEX *in vivo*. Our results showed that DEX reduced OBA and that this effect was reversed in the presence of the sap in mice. The effect of glucocorticoids on the phagocytic responses of phagocytes, including neutrophils, monocytes and macrophages, has been extensively studied, and contradictory results have been reported. Glucocorticoids were found to have an inhibitory effect on ROS production by human monocytes (14), rat peritoneal phagocytes (15) and canine peripheral blood PMNs (13). By contrast, in one study glucocorticoids were reported not to have any effect on the release of reactive oxygen intermediates in cultures of macrophages derived from human blood (16). Another study demonstrated that glucocorticoids increased the phagocytic capacity of human monocytes (14).

To confirm the results of our *in vivo* study, we performed experiments using rat and canine PMNs. The OBA of rat and canine PMNs was increased by addition of the sap. However, the effects of the sap on phagocytic activity differed between the species. In the canine PMNs, the sap enhanced phagocytic activity at a high dose while it did not have any significant effect on rat PMNs.

Oxidative elimination of microbes is essential for the defense mechanism of neutrophils in the innate immune system (17). This process is accomplished through the generation of ROS by phagocyte nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, and its significance is highlighted in cases of chronic granulomatous disease (18). This disease is characterized by oxidative burst-deficient neutrophils with dysfunctional NADPH oxidase (19).

ROS formation, respiratory burst or neutrophil phagocytosis remove pathogens and thereby offer a defense against these microorganisms. However, the production of free radicals, such as ROS, by activated neutrophils at the site of inflammation also inflicts damage on host tissues. Therefore, regulation of neutrophilic OBA is important for maintaining a balance between host tissue injury and the immune defense during the inflammatory process.

The effects of plant extracts on neutrophil function have previously been reported. For example, *Nepeta ucrainica L.*, a herbal tea, was shown to have a positive effect on respiratory bursts in neutrophils (20). By contrast, other plant extracts, including those from *Harpagophytum procumbens*, *Liriope spicata* var. *prolifera* and apples, are believed to inhibit ROS production, immune responses and inflammation (21-23).

In summary, we determined that treatment with *A. okamotoanum* sap stimulated the activity of neutrophils from mice, rat and canines by increasing phagocytic activity and OBA *in vivo* and *in vitro*. These findings suggest that this sap may have potential antimicrobial effects on the PMNs of patients with infection.

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