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In vivo 및 *in vitro* 시험을 통한 페그마타이트의 항염증 효과

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The Anti-Inflammatory Effect of Pegmatite by *in Vivo* and *in Vitro* Study

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Purpose: This work aimed to elucidate the anti-inflammatory effect of pegmatite *in vitro* and *in vivo.*

Methods: Author evaluated the suppressive effects of pegmatite on lipopolysaccharide (LPS)-stimulated nitric oxide (NO) production, TNF-α and IL-6 release in the RAW 264.7 murine macrophages.

Results: Treatment of RAW 264.7 cells with pegmatite significantly reduced LPS-stimulated NO production and inflammatory cytokine such as TNF-α and IL-6 secretion in a concentration-dependent manner. Also pegmatite showed topical anti-inflammatory activity in the arachidonic acid (AA)-induced ear edema and acetic acid-induced increase in capillary permeability assessment in mice. It was also found that pegmatite (10 mg per ear in DW) inhibited arachidonic acid induced edema at 24 h more profoundly than 1 h by topical application. Furthermore, the vascular permeability increase induced by acetic acid was significantly reduced in mice that received pegmatite in 50 mg per mouse.

Conclusion: Therefore the results of the present study suggest that pegmatite is a potent inhibitor of the LPS-induced NO and inflammatory cytokine in RAW 264.7 macrophages and showed anti-inflammatory activities in vivo animal model.

Key Words: Pegmatite, Anti-inflammatory activity, Ear edema, Vascular permeability, NO, LOX

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I. INTRODUCTION

Pegmatite is a very coarse-grained igneous rock that has a grain size of 20 mm or more; such rocks are referred to as pegmatitic. Most pegmatites are composed of quartz, feldspar and mica in essence a "granite". Rarer "intermediate" and "mafic" pegmatite containing amphibole, Ca plagioclase feldspar, pyroxene and other minerals are known, found in recrystallized zones and apophyses associated with large layered intrusions.[1] A mineral is used for offensive odor treatment, improvement of water quality, health and bio-function, enhancement of medical effectiveness. Recently, stones contain trace far infrared ray such as selenium (Se), cerium (Ce), holmium (Ho), germanium (Ge), sodium (Na) were well known to prominent effect in human as well as offensive odor removal and antibacterial effects. Especially, pegmatite consists of various beneficial components not only Ca, K, Mg, Fe but also Se, Ge, Ho. Pegmatite not only promotes growth but also taste and quality of animal and plant. In addition, pegmatite solution is used for prevention and therapeutics of fish diseases and solves to pollution of agriculture water.

In particular, selenium among components of pegmatie is an essential micronutrient that suppresses the redox-sensitive transcription factor NF-B-dependent pro-inflammatory gene expression. In fact, selenium supplementation of macrophages decreased the expression of two pro-inflammatory genes, cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase, via the inactivation of NF-B, whereas the expression of COX-1 was unaffected. Also germanium-concentrated yeast significantly inhibited carrageenan-induced oedema in rat paw. Previously, it has been reported that spirogermanium, an organic germanium, decreases hindleg inflammatory lesions of adjuvant arthritic rats.

Inflammation is a pathophysiological response of mammalian tissues to a variety of hostile agents including infectious organisms, toxic chemical substances, physical injury or tumor growth leading to local accumu-

lation of plasmic fluid and blood cells. A normal inflammatory response is self-limiting and involves the down-regulation of pro-inflammatory protein expression, increased expression of anti-inflammatory proteins, and a reversal in the vascular changes that facilitated the initial immune cell recruitment process.[2] During inflammatory response, this complex process mediated by a variety of signaling molecules produced by leukocytes, macrophages, mast cells, platelets, etc. In various inflammatory conditions, lipoxygenase (LOX) and iNOS, are induced in vascular and/or macrophages. Lipoxygenase (LOX) are sensitive to antioxidants, and the most common way of their action may consist in inhibition of lipid hydroperoxide formation due to scavenging of lipidoxy or lipid peroxy-radicals formed the course of enzymic peroxidation. This LOXs comprise of a family of non-heme iron-containing dioxygenases, representing the key enzymes in the biosynthesis of leukotrienes that have been postulated to play an important role in the pathophysiology of several inflammatory and allergic diseases. LOX enzymes have been shown to be expressed in macrophage-rich areas of atherosclerotic lesion.[3] The clinical treatment of inflammatory diseases is dependent on drugs which belong either to the non-steroidal or steroidal chemical therapeutics. Therefore, a need arises for the development of newer anti-inflammatory agents from natural sources with more powerful activity and with lesser side effects as substitutes for chemical therapeutics.

In this report, author have studied the anti-inflammatory activity of pegmatite *in vitro* and *in vivo* using macrophage activity, mouse ear edema and capillary permeability test. Macrophages play a crucial role in the generation of pro-inflammatory molecules like nitric oxide (NO). NO synthesized by the enzyme inducible nitric oxide synthase (iNOS) has been reported as a mediator of inflammation and seems to be involved in both acute and chronic inflammation. After stimulation with bacterial lipopolysaccharide (LPS), many cells including macrophages express the iNOS which is responsible for the production of large amount of NO. This inducible enzyme is one of the essential components of the inflammatory response and is implicated in the pathogenesis of several inflammatory diseases. Griess assay has been used here to determine the inhibition of nitric oxide formation in RAW 264.7 cells induced by LPS They play an important role in inflammatory diseases by producing the cytokines interleukin (IL)-1β and tumor necrosis factor-α (TNF-α), as well as other inflammatory mediators such as prostaglandin.[4] Also mouse ear edema test and vascular permeability was utilized for the screening of anti-inflammatory effects *in vivo*. Inhibition test of mouse ear edema was known to be a simple and reproducible system to find anti-inflammatory agents. However, it should be mentioned that activities expressed in ear edema bioassay are varies depending on the inflammagens and administration routes used. In croton-oil induced edema, steroidal anti-inflammatory drug types of compounds show sensitive results, while nonsteroidal antiinflammatory drug types of compounds and cyclooxygenase (COU), lipoxygenase (LOX) inhibitors are sensitive in arachidonic acid induced edema. Those models in some ways mimic clinical features and pathogenesis of upper respiratory tract inflammation (i.e. pharyngotonsillitis), including vascular permeability test for swollen mucosa. The development of the increase in vascular permeability induced by acetic acid is known to correspond to the early exudative stage of inflammation. Histamine and serotonin are presumed to play an important roles in the first stage of the acetic acid-induced increase in vascular permeabilty whereas in carrageenan edema, these mediators play a less role.[5]

The main objective of the present study is to explore the anti-inflammatory effect of pegmatite in several experimental models, trying to provide pharmacological evidence for its clinical uses.

II. MATERIALS AND METHODS

A. Materials

Pegmatite used in this study obtained from Mungyeong in Korea. Pegmatite contained 65.5% SiO_2 as standard material. In this study, pegmatite was used by sterilization for more 12 h at 180℃ and mixing sample was prepared by mixing of sterile distilled water and viscosity modifier. Mixed sample was stored at 4℃ until use.

B. Methods

RAW264.7 cells were cultured in plastic dishes containing RPMI-1640 (Gobco Ltd., Grand city, NY, USA) medium supplemented with 10% fetal bovine serum (FBS) (Gobco Ltd., Grand city, NY, USA) in a CO_2 incubator (5% CO_2 in air) at 37℃. After 10 - 15 passages, RAW264.7 cells were no longer used for the assays. The effect of pegmatite on cytotoxicity was tested by treating cells with different concentrations of pegmatite in RPMI - 1640 medium.

The tetrazolium dye colorimetric test (MTT test) was used to monitor cell growth indirectly, as indicated by the conversion of the tetrazolium salt to the colored

product, formazan, the concentration of which can be measured spectrophotometrically. Briefly, the RAW264.7 cell numbers were counted by hemocytometer and seeded in 96-well plates (3×10^4 cells/well in 200 µL of complete RPMI 1640 medium) for 18 h and cells were then coincubated with pegmatite (5,000 19. 5 µg/mL) for 24 h. For the MTT (Sigma, St Louis, MO USA) assay, 10 µL of MTT solution (10 mg/mL) was added to each well of a 96-well plate, and incubated for 4 h. The supernatant was removed, and the formazan crystals produced were dissolved in 200 µL of dimethylsulfoxide, and quantified by measuring their optical density at 550 nm using an ELISA reader (Opsys MR, DYNEX. Ltd). The IC_{50} value (concentration evoking 50% inhibition of growth/viability of cells) for pegmatite was calculated.

The amount of nitrite and inflammatory cytokines produced by the mouse macrophage was indicated by the amount that was measured in the RAW264.7 cell culture supernatant. These cells were plated at a density of 2×10^5 cells in a 24-well plate with 1 mL of culture medium and incubated for 18 h. After adherence, the medium in 24-well plates containing RAW264.7 cell was removed and replaced by medium containing the different formulations of pegmatite at various concentrations (625 19. 5 µg/mL) in the presence of a costimulator where stated below. The costimulator was LPS at 500 ng/mL. After 24 h, the medium was removed and kept for assay of TNF-α (BD science, USA), IL-6 (BD science, USA) or nitrite, the latter being measured as an indication of NOS induction. In order to determine the influence of NO, a competitive inhibitor of NOS, L-N-monomethyllamine (NMMA) (80 µM) was added during this phase. In addition, LPS was preincubated with polymyxin B (100 unit) as LPS inhibitor for 24 h at 37℃ as positive control in cytokine assay.

Nitrite accumulation, an indicator of NO production, was measured using the Griess reagent. Briefly, 100 µL aliquots of culture supernatants were mixed with an equal volume of Griess reagent (mixture at 1 : 1 of 0.1% naphthylethylenediamine dihydrochloride and 1% sulphanilamide in 5% H_3PO_4), and incubated at room temperature for 10 min. The absorbance at 550 nm was measured in an automated microplate reader. Nitrite concentration (µM) was calculated from a $NaNO_2$ standard curve.

The amount of TNF-α and IL-6 in the cell-culture supernatant and in serum was measured using an ELISA kit (OptiEIA, BD science). RAW264.7 cells were plated in a 24-well plate at a density of 2×10^5 cells with 1 mL of culture medium and incubated for 18 h and coincubated with 625 19.5 µg/mL of pegmatite in 50 ng/mL LPS for 24 h. The culture supernatants were collected and assayed, according to the ELISA kit manufacturer's instruction to determine the amount of TNF-α and IL-6 that had been released from the cell.

The LOX activity was measured in borate buffer solutions (0.2 M, pH 9.00) as previously described. The increase in absorbance at 234 nm was recorded at 30s intervals for 5 min at 25℃ after the addition of the enzyme, using linoleic acid (134 µM) as a substrate. Positive control was used L-ascorbic acid (50 µg/mL). The final enzyme concentration was 167 U/mL. Each concentration (625 19.5 µg/mL) of pegmatite was added as DMSO solutions (final DMSO conc. 0.05%) and the DMSO alone was added in uninhibited control experiments.

All experiments were carried out on outbred ICR mice of male and female (5 week old weight 16 - 20 g) obtained from Oreint Co., Ltd (Seoul, Korea). The colony was maintained under controlled conditions of temperature (19 - 25℃), humidity (40 - 60%) and a 12 h light-dark cycle with the light intensity of 150 - 300 Lux. The animals were housed in sanitized Polycarbonate cages (200 W × 260 L × 130 H). They had free access to standard mouse food and water. All animals were raised in SPF condition of the Korea Institute of Toxicology, Dong-A university according to Good Laboratory Practices (GLP) guidelines of OECD. For statistical data *in vivo* animal test, we selected five animals each group.

The method described by Romay et al.[6] with some modifications was followed. Inflammation was induced by topical application 10 µL of 2% arachidonic acid (AA) (Sigma, St Louis, MO USA) of both surfaces of the right ear of each mouse. Left ear (control) received the vehicle. Pegmatite was administered topically (10 mg per ear in DW) 1 h and 24 h before arachidonic acid treatment. Two control groups were used: a group with application of arachidonic acid on the right ear and a positive control group that received nimesulide (1 mg per ear in 20 µL acetone). Inflammation was followed for 1 h and 3 h, animals were sacrificed by cervical dislocation. The ear thicknesses were measured using a dial thickness gauge at 1 h and 3 h after the arachidonic acid treatment, and the differences in the thickness were calculated. The degree of ear swelling was expressed as an increase in ear thickness (mm).

After the measurement of the ear thicknesses, ear tissues were extracted at size of 1.5 × 1.5 cm. The ear tissues were fixed in 10% neutral buffered formalin, dehydrated with 50 - 100% ethanol solutions, and embedded in paraffin. Four to five micrometer sections were

cut and stained with hematoxylin-eosin (H & E), and observed using optical microscopy. Pathologically histological findings in those cases not showing swelling of blood vessels and not showing infiltration of blood cells were classified as being a negative response. A positive response was classified into three levels, slight to mild, moderate, severe, according to the degree of swelling of blood vessels, and the level of blood cell infiltration.

To evaluate the inhibitory activity of pegmatite against acute inflammation, an acetic acid-induced vascular permeability test was employed by modifying the method of Whittle. Pegmatite (50 mg per mouse in DW) was topically administered single dose for 1 h and three times for 24 h and then orally administered indomethacin (Sigma, St Louis, MO USA) as positive control. Mice were intravenously injected into tail veins with 4% Evans blue after 10 min and 0.7% acetic acid was intraperitoneally injected. Thirty minutes later, mice peritoneal exudates were collected after being washed with 5 mL of normal saline, and centrifuged at 200 g for 10 min. The absorbance of the supernatant was read at 630 nm with ELISA Reader (OpsysMR, DYNEX. Ltd). The dye content of the exudates was calculated according to the standard curve of Evans Blue.

C. Statistical analysis

One-way analysis of variance (ANOVA), using the SPSS 10.0 software in a windows environment was used to determine any significant difference. The differences were considered to be significant at $p < 0.05$. All the means were shown with their as mean ± standard deviation (SD).

III. RESULTS

A. Effect of pegmatite on cell viability

To identify cell viability of RAW 264.7 cells in a dose dependent manner, RAW 264.7 cells were assessed using the MTT assay. The results of the cell viability assay appear in Table I. The data shows no cytotoxicity with all concentrations up to 312.5 μg/mL of pegmatite and a high concentration (1,250 - 5,000 μg/mL) of pegmatite was shows a few cytotoxicity. Indeed, an IC_{50} value of pegmatite was 5,000 μg/mL. However, because a high concentration (625 - 5,000 μg/mL) of pegmatite a few reduced cell viability, we used 19.5 - 312.5 μg/mL concentrations of pegmatite in the rest of the experiments

B. Inhibition of NO production by pegmatite in LPS-stimulated RAW264.7 cell

Nitric oxide (NO) synthesis by inducible nitric oxide synthase (iNOS) is increased in inflammatory diseases and leads to cellular injury21. We initially examined the inhibitory effects of pegmatite on the production of the inflammatory mediators such as NO. The amount of produced NO was indicated by amount of nitrite (a stable metabolite of NO) that accumulated in LPS-exposed cells in the presence of each concentration of pegmatite. Treatment of RAW 264.7 cells with LPS (500 ng/mL) for 24 h significantly increased the release of NO (19.5 ± 0.5 μM) by approximately 65-fold compared to that of the untreated cells (0.3 ± 0.1 μM) (Fig. 1). Pegmatite reduced NO release in LPS-stimulated cells in a dose-dependent manner, but had no effect on nitric oxide release in the LPS-untreated cells (Fig. 1). In addition, pegmatite had an inhibitory effect on NO production as

Table I. Cytotoxicity of Pegmatite in RAW 264.7 Cell (IC_{50})

Test material	Treatment concentration (μg/mL)	Cell viability (%)	IC_{50} (μg/mL)
Control (DW)	0	100	
Pegmatite	5,000	61	5,000
	2,500	74	
	1,250	79	
	625	86	
	312.5	95	
	156.3	97	
	78.1	99	
	39.1	100	
	19.5	100	

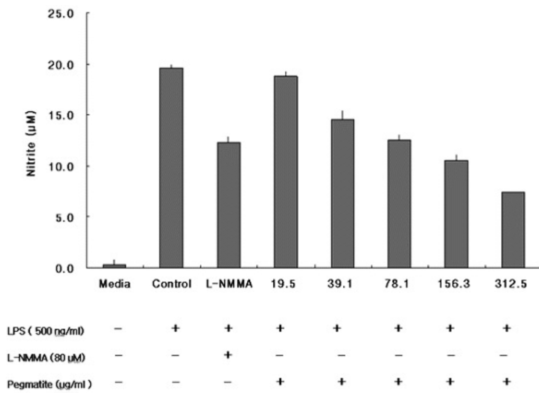

Fig. 1. Inhibition effect of pegmatite on LPS-induced nitirc oxide (NO) in murine macrophages. RAW 264.7 cells were cotreated with concentrations of pegmatite in the presence of 500 ng/mL or with LPS alone for 24 h. L-NMMA (80 μM) as a competitive inhibitor of NOS was added during this phase. The NO concentrations were measured using the Griess reagent. The results are repotrted as mean ± SD of three independent experiments.

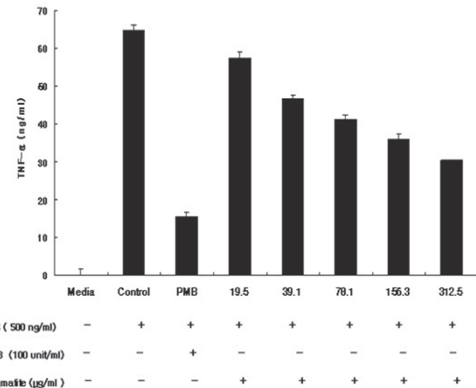

Fig. 2. Inhibition effect of pegmatite on LPS-induced TNF-α in murine macrophages. RAW 264.7 cells were treated with concentrations of pegmatite in the presence of 500 ng/mL or with LPS alone for 24 h. LPS was preincubated with polymyxin B (100 unit) as LPS inhibitor for 24 h at 37℃. The culture media were then collected and the amount of TNF-α released was measured using an ELISA kit. The results are repotrted as mean ± SD of three independent experiments.

strong as that exhibited L-MMNA (12.3 ± 0.3 μM) as a competitive inhibitor of NOS. To confirm that the inhibition of NO release, a cytotoxicity assay for pegmatite (19.5 - 5,000 μg/mL) was examined and treatment at 19.5 - 312.5 μg/mL retained > 90% cell viability in LPS-treated cells (Table I). The present studies also demonstrate that the pegmatite markedly decreased NO production at a low concentration in RAW 264.7 macrophages.

C. Reduced production of proinflammatory cytokines in LPS stimulated RAW264.7 cells

To examine the inhibition effect of pegmatite on proinflammatory cytokine production, RAW264.7 cells were incubated with various concentrations of pegmatite in the presence of LPS for 24 h. TNF-α and IL-6 levels in the culture supernatants were evaluated using ELISA. As shown in (Fig. 2), about 64.8 ng/mL (inhibition : 0%) of TNF-α was secreted from RAW264.7 cells in response to LPS stimulation alone. Specifically, LPS induced TNF-α secretion was reduced to 57.3 - 30.3 ng/mL (inhibition : 11.6 - 55.3%) by 312.5 - 19.5 μg/mL of pegmatite, respectively. Also we found that treatment with pegmatite (312.5 - 19.5 μg/mL) could inhibit IL-6 secretion by 11.8 - 4.6 ng/mL (inhibition : 0 - 58%) as compared with the unpretreated control (10.9 ng/mL, inhibition : 0%) in LPS-stimulated macrophages (Fig. 3). When polymyxin (PMB) as LPS inhibitor was used, LPS-induced inflammatory cytokine (TNF-α and IL-6) was a strongly reduced. Furthermore, pegmatite reduced TNF-α and IL-6 levels in LPS-stimulated cells in a dose-dependent manner and no

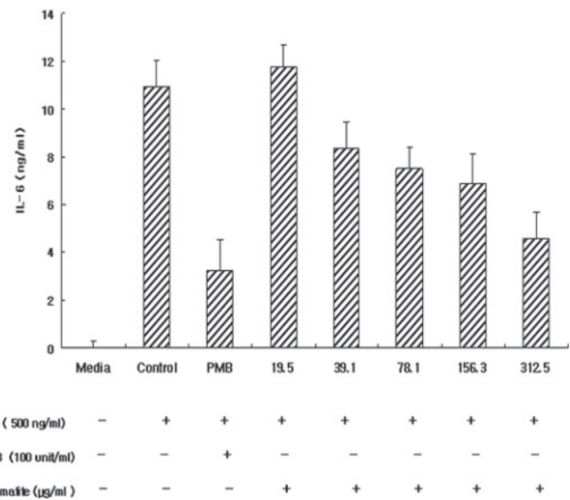

Fig. 3. Inhibition effects of pegmatite on LPS-induced IL-6 in murine macrophages. RAW 264.7 cells were treated with concentrations of pegmatite in the presence of 500 ng/mL or with LPS alone for 24 h. LPS was preincubated with polymyxin B (100 unit) as LPS inhibitor for 24 h at 37℃. The culture media were then collected and the amount of IL-6 released was measured using an ELISA kit. The results are repotrted as mean ± SD of three independent experiments.

cytotoxic effect was observed after cells were treated with various concentrations (19.5 - 312.5 μg/mL) of pegmatite for 24 h as measured by MTT assay.

D. LOX inhibitory activity of P. pini (CY001) fractions

In this study, we elucidated the possible contribution

to the lipoxygenase inhibitory activity of pegmatite. The LOX activity was monitored as an increase in the absorbance at 234 nm, which reflects the formation of hydroperoxylinoleic acid. When tested for inhibition of the enzyme LOX *in vitro*, which peroxidizes polyunsaturated fatty acids, such as linoleic acid to their respective hydroperoxy derivatives, pegmatite showed inhibitory activity of LOX. The results were shown (Table II). Clearly, pegmatite significantly inhibited the LOX-catalyzed oxidation of linoleic acid at 50 μg/mL dose *in vitro* ($p < 0.05$), and this was induced concentration-dependent inhibitory activity of LOX.

E. Arachidonic acid (AA)-induced ear edema and histological changes

The *in vivo* model of arachidonic acid (AA) mouse ear inflammation is very suitable and sensitive. Because the direct topical application of arachidonic acid results in the rapid onset of edema formation. Topical anti- inflammatory activity of pegmatite was evaluated as the inhibition of the AA-induced ear edema in mice. Topical application of AA-induced cutaneous inflammation at the ears of mice, which caused increase in ear thickness of the right ear when compared to the vehicle-treated left ear. Nimesulide (1 mg/ear) as a positive control and pegmatite (10 mg/ear) were topically applied one time for 1 h or 24 h and treatment of arachidonic acid was at 1 h or 3 h. Pegmatite (10 mg per ear in DW) was administered topically before treatment of arachidonic acid and mice were sacrificed to analyse ear thickness. The inhibition effect of ear edema was measured at 1 h or 3 h. When pegmatite was applied topically for 1 h before treatment of arachidonic acid (1 h or 3 h), this a little attenuated the AA-induced edema as compared to the only AA-treated group (Fig. 4). While this was administered topically for 24 h before treatment of arachidonic acid (1 h or 3 h), pegmatite induced effective inhibition effect of

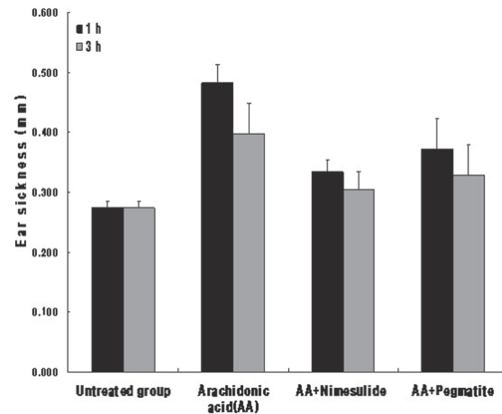

Fig. 4. Inhibition effect of AA-induced ear edema by topical application for 1 h of pegmatite. A group with application of AA on the right ear and a positive control group that treared with nimesulide (1 mg per ear in 20 μL acetone). Pegmatite was applied topically (10 mg per ear in DW) for 1 h before treatment of arachidonic acid (AA) at 1 h or 3 h. The ear thicknesses were measured using a dial thickness gauge at each time (1 h or 3 h) after arachidonic acid treatment. The data are expressed as mean ± SEM of five animals.

ear edema as compared to the only AA-treated group and especially induced significantly different at 1 h of treatment of arachidonic acid (Fig. 5). Elsewhere nimesulide gave rise to inhibition of ear edema at 24 h as only AA-treated group. Indeed, these results presents that pegmatite was effective 24 h than 1 h in topical application before treatment of arachidonic acid at 1h (Fig. 6).

Histological observation was revealed by epidermal hyperplasia and vasodilator of blood cell infiltration by the AA-induced edema. By histological comparison, pegmatite was found to considerably reduce vasodilatoin topical application for 24 h, but epidermal hyperplasia did not show decrease. Also pegmatite treated group was similar to nimesulide treated group (positive control) in effective inhibition effect of ear edema as compared only

Table II. Effect of Pegmatite on LOX Acitivity and LPS-induced Nitric Oxice (NO) Production of RAW 264.7 Cells

Test materials	Treatment (μg/mL)	[a]Inhibition LOX activity (%)	[a]NO inhibition (%)
Control(DW)	0	0	0
L-ascorbic acid	50 μg/mL	30.81 ± 3.41	-
	312.5	60.95 ± 7.58*	62.5 ± 1.38**
	156.3	46.21 ± 6.51*	46.7 ± 7.32**
Pegmatite	78.1	33.31 ± 5.99*	36.8 ± 4.03**
	39.1	15.16 ± 4.06*	27.0 ± 8.45**
	19.5	8.30 ± 2.73	5.6 ± 1.62

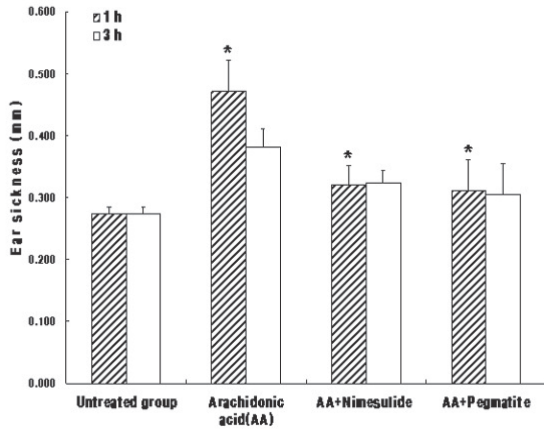

Fig. 5. Inhibition effect of AA-induced ear edema by topical application for 24 h of pegmatite. A group with application of AA on the right ear and a positive control group that treated with nimesulide (1 mg per ear in 20 μL acetone). Pegmatite was applied topically (10 mg per ear in DW) for 24 h before treatment of arachidonic acid (AA) at 1 h or 3 h. The ear thicknesses were measured using a dial thickness gauge at each time (1 h or 3 h) after arachidonic acid treatment. The data are expressed as mean ± SEM of five animals. *Significantly different from only AA treatment group, $p < 0.05$.

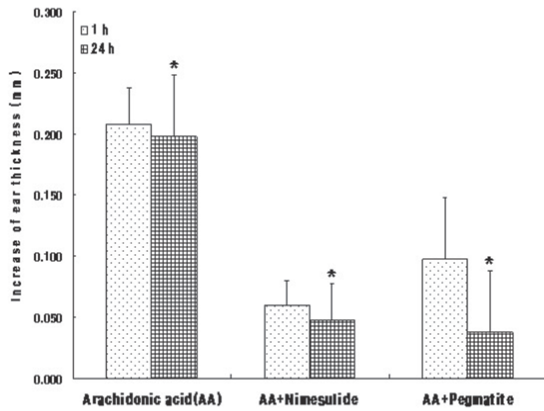

Fig. 6. Inhibition effect by treatment time of pegmatite on AA-induced ear edema. Pegmatite was applied topically (10 mg per ear in DW) 1 h and 24 h before AA treatment and a group with application of AA on the right ear and a positive control group that treated with nimesulide (1 mg per ear in 20 μL acetone). The ear thicknesses were measured using a dial thickness gauge at 1 h after arachidonic acid treatment. The data are expressed as mean ± SEM of five animals. *$p < 0.05$ vs AA group. *Significantly different from only AA treatment group, $p < 0.05$.

AA-treated group (Fig. 7).

Therefore, we suggested that pegmatite was very effective reduction of arachidonic acid (AA)-induced ear edema

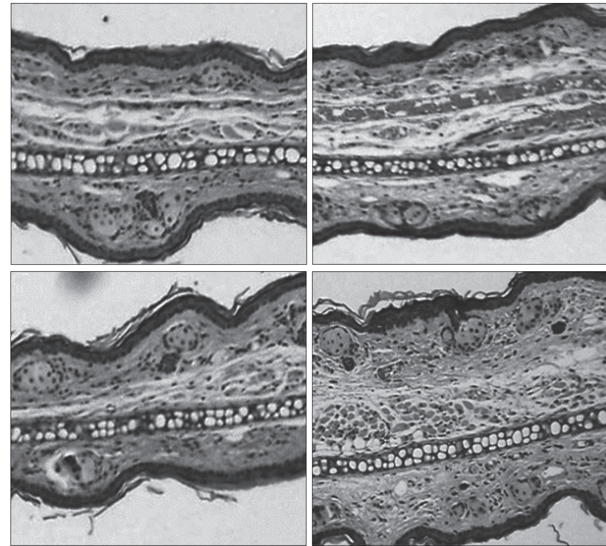

Fig. 7. Histologic finding of anti-inflammatory effect of pegmatite on AA-induced ear edema damage of mouse. Pegmatite was applied topically for 24 h before AA treatment at 1 h. (A) control group (untreated group), (B) animals treated with only 2% arachidonic acid, (C) animals topically applied with pegmatite (10 mg per ear in DW) for 24 h before AA treatment, (D) animals topically treated with nimesulide (1 mg per ear in 20 μL acetone) for 24 h before AA treatment. Specimens were stained with hematoxyli and eosin (H&E), magnification 100 ×.

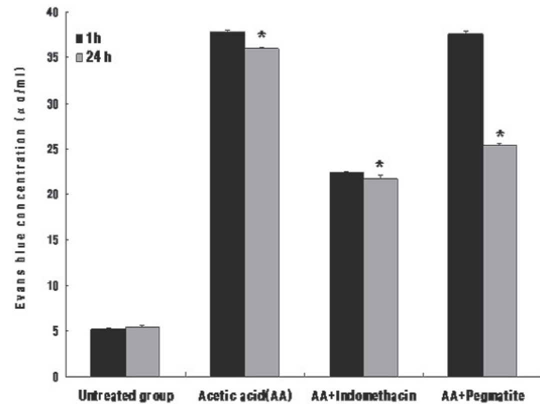

Fig. 8. Inhibition effect of pegmatite on increased vascular permeability induced by acetic acid in mice. Pegmatite (50 mg per mouse in DW) was topically applied single dose for 1 h and three times for 24 h and then orally applied indomethacin as positive control. Results expressed as the increase of Evans blue extravasated are shown as mean SEM of five mice determinations. The data are expressed as mean ± SEM of five animals. *Significantly different from only AA treatment group, $p < 0.05$.

F. Effect on acetic acid-induced increase in vascular permeability

Effect of pegmatite on the increased vascular permea-

bility induced by acetic acid in mice was determined according to Whittle method with some modifications.[7,8] Possibly the pegmatite inhibits more potently the mast cell mediators and affect the vascular permeability increase caused by intraperitoneal acetic acid. When pegmatite (50 mg per mouse) was administered topically for 1 h before treatment of acetic acid, this did not reduce vascular permeability and indomethacin as positive control inhibited 40% of vascular permeability. But pegmatite (50 mg per mouse) was applied topically for 24 h, this inhibited 30% of vascular permeability (Fig. 8). Therefore, vascular permeability inhibition of pegmatite was more effective in animals of topical administration for 24 h than 1 h and significantly suppressed the increase in vascular permeabilty in response to acetic acid.

IV. DISCUSSION

Acute and chronic inflammation is a multiple process, which is mediated by activated inflammatory or immune cells. From them, macrophages play a central role in managing many different immunopathological phenomena such as the overproduction of pro-inflammatory cytokines and inflammatory mediators. A number of inflammatory stimuli such as lipopolysaccharide (LPS) and pro-inflammatory cytokines activate immune cells to up-regulate such inflammatory states and therefore, these are useful targets for developing new anti-inflammatory drugs and exploring the molecular anti-inflammatory mechanisms of a potential drug. The major macrophage derived inflammatory mediators such as proinflammatory cytokines, tumour necrosis factor-α (TNF-α) and the reactive free radical nitric oxide (NO) synthesized by inducible NO synthase (iNOS), contribute to the development of inflammatory diseases. Thus, inhibition of the excessive production of TNF-α and/or NO could be employed as criteria to evaluate potential anti-inflammatory compounds. TNF-α plays a critical role in both acute and chronic inflammation. TNF-α facilitates inflammatory cell infiltration by promoting the adhesion of neutrophils and lymphocytes to endothelial cells. The current management of inflammatory diseases is limited to the use of anti-inflammatory drugs whose chronic administration is associated with several adverse effects. Plant-derived products are slowly emerging as a viable alternative because they are cheap, abundantly available and relatively less toxic.

Author results provide evidence that pegmatite possesses anti-inflammatory activity. The pegmatite was tested with the standard models for inflammation. The *in vitro* test was performed using LPS-stimulated mouse macrophage RAW 263.7 cell model. LPS is antigenic and able to activate monocytes/macrophages to secrete various inflammatory cytokines, including TNF-α, IL-1 α, and IL-6 protein 33-37. Pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6 have been shown to control inflammation *in vitro* as well as *in vivo*, and these cytokines are thought to be interlinked in a cascade, being produced serially by macrophages during an inflammatory response. NO is an important physiological messenger and effector molecule in many biological systems, including immunological, neuronal and cardiovascular tissues. Nitric oxide, in macrophages, is produced as a free radical by iNOS by catalyzing the oxidation of guanidino nitrogen of L-arginine, thereby converting L-arginine to L-citrulline. NO is an important signalling and effector molecule in inflammation and immunity as it is known to couple with superoxides to form peroxynitrite. These, in turn, induce the production of prostaglandin endoperoxide synthase from monocytes/macrophages resulting in enhanced synthesis of prostaglandins, established mediators of inflammation. NO has been found to contribute to many diseases such as inflammation and cancer. In inflammation, macrophages simultaneously produce NO as well as superoxide anion (O_2). The reaction of NO and O_2 generates peroxynitrite anion (ONOO), which can enhance the activity of COXs and therefore stimulates eicosanoids production. Furthermore, peroxynitrite has been found to be a highly reactive oxidant and able to cause DNA damage.[9] Therefore, suppression of NO production by certain phytochemicals might be one of the prominent strategies for the treatment of inflammation and cancer. Activated macrophage can release a series of pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α) or interlukin-6 (IL-6). Therefore, the release of TNF-α or IL-6 after stimulation of RAW 263.7 cell with LPS is a valid model system to test novel compounds for potential anti-inflammatory effects. In the present study, the pegmatite was checked for their inhibitory effect on nitric oxide production from macrophages (RAW 264.7 cells) induced by LPS. Five different concentrations of the pegmatite were used (Fig. 1). This pegmatite had no effects on cytotoxicity showed against macrophages at the higher concentrations and showed a inhibitory effect on nitric oxide in a dose-dependent manner. In addition to, increased TNF-α and IL-6 secretion were observed in RAW 263.7 cell after LPS stimulation and secretion of these pro-inflammatory cytokines was blocked by cotreatment with pegmatite in dose dependent manner.

The processes associated with the inflammatory response are complex but important aspects which have been exploited for screening for anti-inflammatory compounds are the various functions of macrophage and the metabolic products of arachidonic acid.[10] Arachidonic acid release and the resulting formation of eicosanoids are thought to play a critical role in many biological processes such as aging, inflammation, platelet aggregation, angiogenesis, atherosclerosis, and cancers. Many studies have indicated that the inhibition of arachidonic acid metabolism might have potential therapeutic value for cancer prevention.[11] Arachidonic acid is released from cell membranes by phospholipase A2 (PLA2) under the stimulus of several factors associated with inflammation. The products of metabolism of arachidonic acid are collectively known as eicosanoids and the two most important groups are the prostaglandins and leukotrienes, formed by the actions of cyclo-oxygenases (COX) and lipoxygenases (LOX), respectively. To identify relation to metabolism of arachidonic acid *in vitro* test, author tested the LOX activity and the pegmatite appeared to significantly inhibit enzymatic lipid peroxidation mediated by the LOX activity at 50 μg/mL dependent on concentration.

In vivo tests using animal models give the most meaningful results when testing plant extracts for anti-inflammatory activity but they are unsuitable for bioassay-guided fractionation to determine the active compounds present and, in many countries, any use of animals is minimized for economic and ethical reasons. Several models have been developed and used but probably what is the most often reported is the rat hind paw edema model developed by Winter et al.[12] In this study, the anti-inflammatory effects of pegmatite were determined using two different models, i.e., the ear edema induced by arachidonic acid in mouse and the acetic acid-induced vascular permeability in mice. Above all, the author evaluated the topical anti-inflammatory activity of the pegmatite on arachidonic acid (AA)-induced ear edema *in vivo* test. In the (Fig. 7), pegmatite induced a strong reduction of the edematous response, when pegmatite was applied topically for 24 h than 1 h. Considering *in vitro* test and *in vivo* test results, we suggested that the pegmatite would show anti-inflammatory effect related to 5-lipoxygenase pathways of arachidonate metabolism.

Besides, the authors studied *in vivo* anti-inflammatory activity of pegmatite using capillary permeability *in vivo* test. The test is used to evaluate the inhibitory activity of drugs against increased vascular permeability which is induced by acetic acid by releasing inflammatory mediators. Mediators of inflammation, such as histamine, prostaglandins and leukotrienes are released following stimulation of mast cells. This leads to a dilation of arterioles and venules and to an increased vascular permeability. As a consequence, fluid and plasma protein are extravaseted and edemas are formed. The development of the increase in vascular permeability induced by acetic acid is known to correspond to the early exudative stage of inflammation. Histamine and serotonin are presumed to play an important roles in the first stage of the acetic acid-induced increase in vascular permeability whereas in carrageenan edema, these mediators play a less role. Likewise result of AA-induced ear edema, vascular permeability inhibition of pegmatite was more effective in animals of topical application for 24 h than 1 h in response to acetic acid.

V. CONCLUSION

In conclusion, we have demonstrated that anti-inflammatory effect of pegmatite may be able induced macrophage mediated inflammatory function such as the over-production of cytokines and NO. Moreover pegmatite showed an inhibitory effect on edema induced by arachidonic acid and vascular permeability increased by acetic acid in animal model. The results may help to provide a scientific basis for its popular use as an anti-inflammatory agent. Further studies will be undertaken to elucidate the mechanism of action by which the pegmatite exert their anti-inflammatory activity.

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