Protective effect of boiogito extract with glucosamine HCl against adjuvant-induced arthritis in rats

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ABSTRACT

Aim: The effect of boiogito extract combined with glucosamine HCl was assessed in adjuvant-induced arthritis (AIA) rats. Methods: Rats received a daily oral mixture of boiogito extract (125 mg/kg) and glucosamine HCl (80 mg/kg) before and after once-off adjuvant injection. Treatment was continued up to the day when blood and/or tissue were collected (day 12 or day 21). Paw swelling, arthritis score, and inflammatory mediators were assessed. Results: Combined treatment was more effective for AIA than either agent alone. Both serum nitric oxide (NO) and tissue NO were significantly suppressed in the adjuvant-uninjected hind paw on day 12. Boiogito extract significantly reduced the tissue level of matrix metalloproteinase (MMP)-13. In addition, interleukin (IL)-1β-induced MMP-13 by SW1353 chondrocytes was dose-dependently suppressed by boiogito extract, while glucosamine HCl had little effect. IL-1β-induced phosphorylation of extracellular signal-related kinase (ERK) was inhibited by the combination of boiogito extract and glucosamine HCl, but was most strongly suppressed by glucosamine HCl alone. Conclusion: Combined treatment with boiogito extract and glucosamine HCl had a protective effect against AIA in rats. This effect was related to strong suppression of MMP-13 production (mainly by boiogito extract) and inhibition of inflammatory mediator production via ERK signaling (mainly by glucosamine HCl).

KEY WORDS: boiogito, glucosamine HCl, matrix metalloproteinase-13, osteoarthritis

INTRODUCTION

Osteoarthritis (OA) is one of the most common chronic diseases and is a major cause of disability in the elderly. Its incidence is increasing rapidly as the world’s population ages. OA is characterized by cellular loss in articular cartilage, an important factor in progressive cartilage deterioration, given that apoptosis of chondrocytes leads to degradation of the extracellular matrix (ECM) in the joint cartilage of OA patients [1]. Matrix metalloproteinases (MMP) play a critical role in the initiation and progression of cartilage destruction together with various cytokines [2,3].

Boiogito is a traditional Japanese herbal medicine containing boi (stem or rhizome of Sinomenium acutum (Thunb.), ogi (root of Astragalus membranaceus Bunge), sojutsu (rhizome of Atractylodes lancea DC.), taiso (fruit of Zizyphus jujuba Miller var. inermis Rehder), kanzo (root of Glycyrrhiza uralensis Fisch. ex DC.), and sho-kyo (rhizome of Zingiber officinale Roscoe). In Japan, boiogito has been used clinically for the treatment of edema, arthritic pain, and nephrotic syndrome. Kogure et al. reported that treatment with boiogito improved both the Harris hip score and clinical criteria in OA patients [4]. In addition, boiogito has been shown to have a cardioprotective effect in the isolated blood-perfused canine heart [5], to inhibit obesity in ovariectomized rats [6], and to increase fatty acid metabolism by cells of the proximal renal tubules [7]. Furthermore, boiogito has been reported to improve hydrarthrosis in a rat model of knee OA [8].

Glucosamine has been shown to be effective for adjuvant arthritis in rats [9–11]. In addition, Nakamura et al. showed that glucosamine significantly improved pain and swelling of arthritic joints compared with placebo in patients with rheumatoid arthritis (RA) [12]. Matsuno et al., however,
concluded that glucosamine was more effective for OA than RA [13], and Kulkarni et al. reported that glucosamine HCl improved pain in 59 patients with knee OA [14]. Recently, a randomized, double-blind, placebo-controlled trial by Sterzi et al. showed that glucosamine HCl combined with chondroitin sulfate and bio-curcumin was effective for knee OA [15].

Locofit GL® (Ohta’s Isan, Tokyo, Japan) contains both boiogito extract and glucosamine HCl and has been used to treat hyperhidrosis or arthritic joint swelling and pain associated with obesity in Japan. The efficacy of this combination of boiogito extract and glucosamine HCl, however, has not been evaluated. The aim of this study was therefore to assessed the effect of this combined product in rats with adjuvant-induced arthritis (AIA), and compare it with the effects of boiogito extract or glucosamine HCl alone.

**METHODS**

**Reagents**

Boiogito extract (boiogito dried extract-O, Lot No. 10 K176) was provided by Alps Pharmaceutical Industries (Gifu, Japan). Boiogito extract was prepared as follows. The following materials were extracted with 20 volumes of distilled water for 50 min at 95–100°C: boi (X3F138), ogi (E3H176), sojutsu (E3D81), taiso (E4C52), kanzo (S15F119), and shokyo (S15G131) (5.0/5.0/3.0/3.0/1.5/1.0; w/w; Table 1). The solution was filtered and concentrated at 60°C in vacuo, followed by spray drying (yield: 17.2%). The extract thus obtained contained 0.77% glycyrrhizic acid and 0.39% sinesis (Figure S1 and S2).

Glucosamine HCl was purchased from Sigma-Aldrich (St Louis, MO, USA).

**Cell lines**

Chondrosarcoma cell line (SW1353) was purchased from ATCC (Salisbury, UK). Cells were cultured in D-MEM (Sigma-Aldrich) with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and 1% antibiotic/antimycotic (Thermo Fisher Scientific, Waltham, MD, USA).

**Animals**

Wistar rats were purchased from Charles River Laboratories Japan (Yokohama, Japan). The rats were housed in an animal room at a constant temperature (22–24°C) and humidity (50–60%) with a 12 h light/dark cycle, and were allowed free access to a standard diet and water. The animal experiments were performed in accordance with the Guidelines for Animal Experimentation of St Marianna University Graduate School of Medicine (approval no. 1501010).

**AIA model**

Seven-week-old female rats (215.7 ± 11.5 g) were randomly divided into the following groups (n = 5 each): (i) saline-treated AIA control group; (ii) combined therapy (boiogito extract 125 mg/kg and glucosamine HCl 80 mg/kg) AIA group; (iii) boiogito extract (125 mg/kg)-treated AIA group; (iv) glucosamine HCl (80 mg/kg)-treated AIA group; and (v) a normal control group. Complete Freund’s adjuvant (0.5 mg/0.05 mL, Chondrex, Redmond, WA, USA) was injected once, intradermally, into the right hind foot pad. Test compounds suspended in saline (1 mL/kg) were given orally at 1 h before the adjuvant injection, followed by daily treatment up to day 12 or day 21. Although the daily dose of Locofit GL® is 3.6 g in humans (approx. 60 mg/kg), a fivefold higher dose was used in this study, corresponding to 125 mg/kg for boiogito extract and 80 mg/kg for glucosamine HCl.

Paw volume was determined every 3 or 4 days with a plethysmometer (TK-101CMP; Unicom, Chiba, Japan). Paw erythema and swelling were then graded as per the following 5-point scale, and arthritis score [16] calculated: 0, no inflammation; 1, swelling and erythema of the digits; 2, moderate swelling and erythema of the paw; 3, severe swelling and erythema of the limb; and 4, severe swelling, erythema, gross deformity, and difficulty using the limb.

**Serum measurements and uninjected paw**

After in vivo observation, a blood sample was collected under anesthesia, and serum was stored. On day 12, the adjuvant-uninjected hind paw was resected at the ankle under deep anesthesia. Paw tissue samples were homogenized in 20 mmol/L Tris–HCl (pH 7.5), 2 mol/L NaCl, 0.1% Tween-80, 1 mmol/L phenylmethylsulfonylfluoride (PMSF), and 1% 100–600 KDa Dextran (Pharmacia, Uppsala, Sweden). After centrifugation at 12,000 × g and 4°C for 10 min, the supernatant was used for analysis. Serum measurements and uninjected paw sections were isolated and used for polymerase chain reaction (PCR) analysis.

**Table 1** | Components of boiogito

| Original plant | Plant part | Family name | Japanese name |
|----------------|-----------|-------------|---------------|
| *Sinomenium acutum* (Thunb.) Rehder & E.H. Wilson | Stem, rhizome | Menispermaceae | Boi |
| *Astragalus membranaceus* Bunge | Root | Leguminosae | Ogi |
| *Atractylodes lancea* DC. | Rhizome | Compositae | Sojutsu |
| *Zizyphus jujuba* Miller var. *inermis* Rehder | Fruit | Rhamnaceae | Taisu |
| *Glycyrrhiza uralensis* Fisch. ex DC. | Root | Leguminosae | Kanzo |
| *Zingiber officinale* Roscoe | Rhizome | Zingiberaceae | Shokyo |

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and 1 mmol/L ethylenediaminetetra-acetic acid (EDTA), followed by centrifugation.

Hyaluronic acid (HA), nitric oxide (NO), prostaglandin (PG)E₂, and MMP-13 were measured with an HA assay kit (Seikagaku, Tokyo, Japan), nitrate/nitrite assay kit, PGE₂ ELISA kit (Cayman Chemical, Ann Arbor, MI, USA) and MMP-13 ELISA kit (R&D Systems, Minneapolis, MN, USA), respectively.

**Chondrocyte activity**

SW1353 cells (3 × 10⁵) were cultured for 24 h in serum-free medium, incubated for 2 h with boiogito extract and/or glucosamine HCl, and then cultured in fresh medium containing IL-1β (10 ng/mL) for a further 24 h. Culture supernatants were used for measurement of MMP-13 and tissue inhibitor of matrix metalloproteinase (TIMP)-1 (R&D Systems). In addition, cells (2 × 10⁶) incubated for 2 h with reagents were exposed to IL-1β for 15 min, after which phosphorylation of extracellular signal-related kinase (ERK) and p38 was assessed.

Boiogito extract and glucosamine HCl were dissolved in phosphate-buffered saline and sterilized by passage through a 0.45 μm membrane filter.

**Western blotting**

Lysates were prepared with 20 mmol/L Tris–HCl (pH 7.5), 150 mmol/L NaCl, 1(v/v)% Triton X-100, 5 mmol/L EDTA, 1 mmol/L PMSF, 5 μg/mL aprotinin, 5 μg/mL leupeptin, 2 mmol/L imidazole, 1 mmol/L sodium fluoride, 1.15 mmol/L sodium molybdate, 1 mmol/L sodium orthovanadate, and 4 mmol/L sodium tafrate dihydrate.

After centrifugation, the lysates (20 μg) were separated by electrophoresis on 10% sodium dodecylsulfate polyacrylamide gels and proteins were transferred to polyvinylidene difluoride membranes. The membranes were incubated with antibodies targeting p44/42 mitogen-activated protein kinase (MAPK; ERK, rabbit; Cell Signaling Technology, Danvers, MA, USA), phosphor-p44/42 MAP kinase (p-ERK, rabbit; Cell Signaling Technology), phosphor p38 (mouse; Abcam, Cambridge, UK), or p38 (rabbit, Cell Signaling Technology), followed by incubation with electrochemiluminescence (ECL) horseradish peroxidase (HRP)-labeled anti-mouse IgG or HRP-labeled anti-rabbit IgG (GE Healthcare Japan, Tokyo, Japan). Then the membranes were incubated with the ECL Prime Western blotting detection system (GE Healthcare Japan) and signals were quantified on densitometry.

**Statistical analysis**

Data are given as mean ± SEM. One-way analysis of variance followed by Tukey test or Dunnett’s test was used to evaluate differences, which were considered to be significant for P < 0.05.

**RESULTS**

**Hind paw swelling and arthritis score**

In saline-treated AIA control rats, rapid swelling of the right hind paw was noted after adjuvant injection and there was a subsequent steady increase of paw swelling until day 21 (Fig. 1a). In the combined boiogito extract and glucosamine HCl (Ext + GlcN)-treated AIA group, however, significant reduction of hind paw swelling was achieved during the observation period.

In the AIA control group, swelling of the adjuvant-uninjected left hind paw was detected on day 12 and increased until day 21 (Fig. 1b). In the AIA control, the mean increase of paw volume was >1.0 mL. In the Ext + GlcN AIA group, however, the increase in paw volume was significantly suppressed.

In the AIA control, the arthritis score increased to 7.80 ± 1.38 by day 21 (Fig. 1c), while in the Ext + GlcN AIA group, arthritis score was significantly reduced. Boiogito extract alone also reduced the score, but it was not significant; and glucosamine HCl alone had little effect. Thus, combined treatment (Ext + GlcN) was more effective than either agent alone for alleviating the signs and symptoms of AIA.
Serum HA was significantly elevated in the AIA control compared with the normal control (Fig. 1d), while in the combined treatment group the increase was significantly suppressed.

Next, we focused on day 12, which was when the volume of the adjuvant-uninjected hind paw began to increase. Serum HA was slightly elevated in the AIA control. The increase was suppressed in all other treatment groups, but the effect was not significant (Fig. 2a).

In the AIA control, serum NO was dramatically elevated to 48.7 ± 7.5 μmol/L on day 12 (Fig. 2b). In the combined treatment AIA group, serum NO was reduced to 27.7 ± 3.1 μmol/L. Both boigotio extract alone and glucosamine HCl alone suppressed serum NO, but neither agent had a significant effect.

Tissue NO was significantly higher in the AIA control compared with the normal control (32.3 ± 3.1 nmol vs 5.4 ± 0.3 nmol per hind paw). Combined treatment lowered the increase (Fig. 2c). Glucosamine HCl suppressed the NO, but the boigotio extract had little effect.

Tissue PGE2 was significantly higher in the AIA control than in the normal control group (4.21 ± 0.90 ng vs 1.64 ± 0.28 ng per hind paw). PGE2 was lowered in all treatment groups, but this was not significant (Fig. 2d).

Tissue MMP-13 was significantly increased in the AIA control than in the normal control group (27.9 ± 1.4 ng vs 16.6 ± 2.1 ng per hind paw; Fig. 2e). It was significantly lower in the boigotio extract-treated group.

**SW1353 chondrocyte MMP-13 and TIMP-1 production in vitro**

When chondrocytes were exposed to IL-1β, there was a significant increase in MMP-13, while boigotio extract caused
Hyaluronic acid is an inflammatory mediator [17], and its level was significantly decreased by boiogito extract. Both serum and tissue (adjuvant-uninjected hind paw) NO were also significantly suppressed by boiogito extract. Furthermore, tissue PGE2 was reduced by boiogito extract, although not significant. This suggests that suppression of inflammatory mediators contributes to the anti-arthritic activity of boiogito extract.

Glucosamine has already been reported to be effective in AIA rats [9–11]. Following oral treatment, glucosamine is absorbed and metabolized, after which it may be incorporated into arthritic joints and support chondrocyte function [18]. Nakamura et al. reported that glucosamine reduced joint pain and swelling in RA patients [12], while Matsuno et al. concluded that glucosamine was more effective for OA than RA [13]. A recent randomized, double-blind, placebo-controlled study showed that glucosamine HCl combined with chondroitin sulfate and bio-curcumin was effective for knee OA [15]. The equivalent dose of glucosamine HCl alone, however, had little influence on AIA in the present rat model. Therefore, it seems that glucosamine-HCl itself was not effective against AIA in the present study, but acted to promote the pharmacological effects of boiogito extract.

The TIMP regulate tissue breakdown by blocking the activity of MMP, and the balance between these enzymes is assumed to be a critical determinant of ECM turnover, especially during progression of OA. Boiogito extract significantly reduced tissue MMP-13 in the adjuvant-uninjected hind paw. Boiogito extract also suppressed IL-1β-induced MMP-13 production by SW1353 chondrocytes, while increasing TIMP-1, suggesting that not only a decrease of MMP-13 but also reduction of the MMP-13/TIMP-1 ratio may play an important role in suppressing paw edema in vivo. Glucosamine HCl had little effect on MMP-13 and TIMP-1 by chondrocytes.

The MAPK participate in inflammation and joint destruction in both RA and OA, and MAPK inhibitors have been reported to decrease synovial inflammation and cartilage damage in an animal model of arthritis [19]. MAPK are also activated by treating cultured chondrocytes with IL-1β [20]. Lu et al. found that MMP-13 was suppressed by inhibiting the phosphorylation of MAPK such as ERK and p38 [21]. In the present study, boiogito extract suppressed ERK phosphorylation in association with a decrease of MMP-13, while glucosamine HCl markedly suppressed ERK phosphorylation with little effect on MMP-13. Scotto d’Abusco et al. reported that glucosamine HCl suppressed MMP-13 production by inhibiting the phosphorylation of p38, but not ERK [22], which is inconsistent with the present results. Although further investigation of this issue is required, the present results

### Table 2 | Effect of boiogito and/or glucosamine on IL-1β-induced MMP-13 and TIMP-1 SW1353 cell production

| Boiogito extract | Glucosamine HCl | IL-1β | MMP-13 (ng/mL) Mean ± SEM | TIMP-1 (ng/mL) Mean ± SEM | MMP-13/TIMP-1 ratio |
|------------------|-----------------|-------|---------------------------|---------------------------|---------------------|
| (−)              | (−)             | (−)   | −0.05 ± 0.02              | 19.5 ± 0.81               | (−)                 |
| (−)              | (−)             | (+)   | 45.8 ± 1.05               | 21.4 ± 0.93               | 2.16 ± 0.09         |
| (+)              | (+)             | (+)   | 40.5 ± 0.46*†             | 26.8 ± 1.23*†             | 1.60 ± 0.07**†      |
| (+)              | (−)             | (+)   | 40.3 ± 0.77*†             | 26.8 ± 1.43*†             | 1.73 ± 0.14**†      |
| (−)              | (+)             | (+)   | 45.4 ± 1.50               | 24.1 ± 1.43               | 1.90 ± 0.08         |

* P < 0.05; ** P < 0.01; † vs control (IL-1β alone).

Final concentration was 3 mg/mL for boiogito extract and 10 mmol/L for glucosamine HCl.

IL, interleukin; MMP, matrix metalloproteinase; TIMP-1, tissue inhibitor of matrix metalloproteinase.
suggest that ERK signaling might be involved in MMP-13 production by chondrocytes, but glucosamine HCl seems to have little influence on MMP-13 despite suppressing the ERK pathway. Kim et al. and Jang et al. reported that suppression of ERK had an anti-inflammatory effect [23,24], and glucosamine has been reported to alleviate oxidative stress by inhibition of MAPK signaling [25]. In the present study, both serum and tissue NO were lower on day 12 in rats treated with glucosamine HCl, although the differences were not significant. Therefore, although glucosamine HCl had little influence on MMP-13, it may have had an anti-inflammatory effect via the ERK pathway that contributed to greater in vivo reduction of NO.

In conclusion, the combination of boiogito extract and glucosamine-HCl could be an effective treatment for joint inflammation in diseases such as OA. In addition to strong suppression of MMP-13 production by boiogito extract, the anti-inflammatory effect of glucosamine HCl mediated via ERK signaling suggests the more potent anti-arthritic activity of this combination.

It is also important to determine which component of boiogito extract has the most potent anti-arthritic activity. Sinomenine, a major component of boi, has been reported to ameliorate arthritis by acting on MMP [26,27]. The doses of sinomenine tested in this study were equivalent to 0.48 mg/kg (125 mg/kg in vivo) and 32 μmol/L (3 mg/mL in vitro), being much lower than in previous reports. Although the data are not shown, 50 μmol/L sinomenine alone had a weak inhibitory effect, but a high dose (250 μmol/L) had similar efficacy to that of boiogito extract (Table 2). Therefore, other components of boiogito extract must have contributed to its activity in addition to sinomenine.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Supplementary Material.

Figure S1 Glycyrrhizic acid.

Figure S2 sinomenine in boiogito extract.