

RESEARCH ARTICLE

Caffeic Acid Inhibits Swelling, Bone Loss, and Osteoclastogenesis in Adjuvant-induced Arthritis Rats

Ferry Sandra^{1,2,*}, Muhammad Ihsan Rizal¹, Nurrani Mustika Dewi³, Toshio Kukita²

¹Department of Biochemistry and Molecular Biology, Division of Oral Biology, Faculty of Dentistry, Universitas Trisakti, Jl. Kyai Tapa No.260, Jakarta, Indonesia

²Department of Molecular Cell Biology and Oral Anatomy, Faculty of Dental Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

³The Prodia Education and Research Institute, Jl. Kramat Raya No.150, Jakarta 10430, Indonesia

*Corresponding author. E-mail: ferry@trisakti.ac.id

Received date: Aug 12, 2022; Revised date: Aug 22, 2022; Accepted date: Aug 26, 2022

Abstract

BACKGROUND: Increase in inflammatory cytokine levels promotes pathological osteoclast differentiation. Caffeic acid has anti-inflammatory properties and can inhibit osteoclast bone resorption. *In vitro* studies have reported the ability of caffeic acid in inhibiting osteoclastogenesis pathways, however the *in vivo* study is rarely conducted. The aim of this study is to examine the role of caffeic acid in reducing inflammation and inhibiting osteoclastogenesis in Adjuvant-Induced Arthritis (AIA) rats.

METHODS: Rats were injected with Freund's Complete Adjuvant (CFA) and mineral oil. One day after injection, various concentration (0, 5, 25, 125 mg) of caffeic acid were given gastro-intestinally. Swelling degree in rats' ankle joints was determined by measuring height and width of each ankle joint. Bone loss level was examined with soft X-ray, and then bone density was calculated. To examine osteoclastogenesis, ankle joints were stained with

Tartrate-Resistant Acid Phosphatase (TRAP) and evaluated microscopically.

RESULTS: Ankle joints of AIA rats had severe swelling before treated, yet the swelling was reduced based on concentration-dependent after receiving caffeic acid. Severe bone loss in AIA rats' ankle joints were also observed, however the treatment of 125 mg caffeic acid showed remarkable inhibition effect toward rats' bone loss. Osteoclastogenesis in AIA rats' ankle joints were higher than the normal ones, as indicated with high TRAP-positive Multinucleated Cells (MNCs). But low number of TRAP-positive MNCs was observed in ankle joint of AIA rats that received 125 mg caffeic acid.

CONCLUSION: Administration of caffeic acid can reduce the degree of swelling, inhibit bone loss, and inhibit osteoclastogenesis in ankle joint of arthritis-induced rats.

KEYWORDS: caffeic acid, osteoclastogenesis, bone loss, swelling, inflammation, RANKL, TNF- α

Indones Biomed J. 2022; 14(3): 276-81

Introduction

Increase in inflammatory cytokine levels, such as Receptor Activator Nuclear Factor κ B Ligand (RANKL), Macrophage Colony Stimulating Factor (M-CSF), and Tumor Necrosis Factor (TNF)- α , might promotes pathological osteoclast

differentiation that leads to excessive bone resorption.(1,2) Rheumatoid arthritis, including Temporomandibular-Joint (TMJ) osteoarthritis, is an osteopenic disorder that is caused by the increase of osteoclast activity.(2,3) TMJ osteoarthritis is characterized by chronic inflammation in synovial tissue, progressive cartilage destruction and deterioration, and subchondral bone remodelling.(3)

Osteoclasts are Multinucleated Cells (MNCs) originated from the monocyte/macrophage haematopoietic lineage that develop and adhere to bone matrix.(4,5) RANKL and M-CSF are known to be major role as normal supply for osteoclasts.(6) Meanwhile, the bindings of RANKL to RANK and TNF to TNF receptor are able to induces intracellular signalling pathways, including TNF Receptor (TNFR)-associated Factor 6 (TRAF6), Mitogen Activated Protein Kinase (MAPK) family, and Nuclear Factor-kappaB (NF-κB).(7) TRAF6 may be important for RANK signalling in osteoclasts, since a TRAF6 knockout leads to compromised differentiation and defective activation of osteoclasts. TRAF6 is also shown to induce NF-κB activity. (8) RANKL-induced osteoclast differentiation can be blocked by TRAF6-binding decoy peptides in a model cell line and in primary cells.(9)

Osteoclastogenesis is a complicated procedure consist of the commitment, differentiation, multinucleation, and activation of immature osteoclasts.(10) Many efforts had been done to stop osteoclastogenesis, such as by using osteoprotegerin, a decoy receptor for RANKL, which is the most related ligand to induce osteoclastogenesis.(6,11) Other than osteoprotegerin, another chemical reagents that also have the function to inhibit osteoclastogenesis is caffeic acid.(12)

Caffeic acid (3,4-dihydroxycinnamic acids), a major representative of hydroxycinnamic acids, can be found in food mainly as an ester with quinic acid called chlorogenic acid (3-O-caffeoylquinic acid).(13,14) Coffee is the main source of chlorogenic acid, and after coffee intake, chlorogenic will hydrolize to caffeic acid in the gastrointestinal tract.(15) Caffeic acid is a natural resource with abundant functions in medicine. One of its derivatives, Caffeic Acid Phenyl Ester (CAPE), was also known to have potential to inhibit the activation of Nuclear Factor Kappa-B (NF-κB).(6,16) CAPE is a potential therapeutic agent that have anti-inflammatory properties and is able to inhibit osteoclast bone resorption and, thus it could potentially be used for the prevention or treatment of arthritic bone diseases.(17) The inhibition of RANKL function via the decoy receptor osteoprotegerin completely prevents bone loss at inflamed joints and has partially in arthritis rats models.(2)

Some *in vitro* studies have reported the ability of caffeic acid in inhibiting osteoclastogenesis pathways, however the *in vivo* study is rarely conducted. Our previous study reported that caffeic acid from Simon extracts has an activity to inhibit osteoclastogenesis (12), and in the present study, we investigate substantial role of caffeic

acid to reduce swelling/inflammation and to inhibit the osteoclastogenesis in Adjuvant-Induced Arthritis (AIA) rats model.

Methods

Induction of Arthritis in Rats

Injection of AIA was performed based on previous study with slight modification.(12) Five-weeks old female Lewis rats were intradermally-injected with Freund's Complete Adjuvant (CFA), a suspension of 1 mg heat-killed *Mycobacterium butyricum* (Difco, Detroit, MI, USA) and 0.1 mL mineral oil (Sigma-Aldrich, St. Louis, MO, USA), at the base of its tail. One day after the CFA-injection, various concentration (0, 5, 25, 125 mg) of caffeic acid suspension in 1 mL distilled water were given gastro-intestinally using oesophageal tube (Cat No. KN-349B, Nalume, Tokyo, Japan) once per day for 21 days. All animal experiments were performed under the principals of 'Care and Use of Animals' of Kyushu University, Japan.

Degree of Swelling Assessment

The degree of swelling in AIA rats' ankle joints was measured on the 5th, 9th, 13th, 17th and 21st day after the adjuvant injection. The degree of swelling was estimated by measuring height and width of each ankle joint using a digimatic calliper (Mitutoyo, Kawasaki, Japan). Pictures of the rats' ankle joints were also taken on the 21st day after the injection for comparison.

Hard Tissue Radiography

At the 21st day, the level of bone loss was examined by exposing the rats' ankle joints to soft X-ray using Sofron (SRO-M50, Sofron, Tokyo, Japan) for 25kV, 5mA, 30 sec. For calculating bone density, X-ray pictures were inverted, and the density was calculated.

Histology Analysis

For histological study, rats were fixed by perfusion with freshly prepared 4% paraformaldehyde in Phosphate Buffered Saline (PBS). Rat's ankle joints were dissected and decalcified for 3 weeks using 10% Ethylenediaminetetraacetic Acid (EDTA). After decalcification, rats' ankle joints were dehydrated and embedded in paraffin. As much as 4 μm of the sagittal sections of the ankle joints were stained for Tartrate-Resistant Acid Phosphatase (TRAP) using Acid Phosphatase, Leukocyte TRAP Kit (Sigma-Aldrich) and counterstained with methyl green. The paraffin slides were

then incubated for 1 hour at 37°C in the dark before being rinsed and evaluated microscopically.

Statistical Analysis

Results are expressed as the mean±SEM. Analysis was performed using StatView-J5.0 (SAS Institute Inc., Cary, NC, USA). Student's T-test was used to determine the statistical differences between the means of experiments, and p -value<0.05 was considered to be statistically significant.

Results

Caffeic Acid Decreased Swelling in Ankle Joint of AIA Rats

Out of 15 rats, 12 rats were injected with FCA to create AIA rat model, while the other 3 only received mineral oil. The ankle joints of rats receiving no FCA injection were shown to be normal without any swelling (Figure 1A), while the ankle joints of AIA rats had severe swelling before being treated with caffeic acid (Figure 1B). However, the swelling of AIA rats' ankle joints was reduced based on concentration-dependent after receiving caffeic acid (Figure 1C, 1D, and 1E).

The swelling degree of rats' ankle joints was measured in height (Figure 1F) and width (Figure 1G) perspectives on consecutive days as shown in the graphs. The degree of

swelling in height was seen slightly higher than the one in width. Swelling of ankle joints of AIA rats was clearly seen starting on the 9th day. The swelling was increasing in both height and width of the AIA rats' ankle joints and reached the top on the 17th day, then mostly remained in the same size or slightly decreased on the 21st day. The numbers of swelling degree results showed that caffeic acid rendered the swelling enormously, both height- and width-wise, especially for the group of rats that was treated with 125 mg caffeic acid.

Caffeic Acid Inhibited Bone Loss in Ankle Joints of AIA Rats

Following the degree of swelling assessment, the severe bone loss in AIA rats' ankle joints were observed based on the inverted X-ray pictures. The bone density in rats that received only mineral oil was shown to be normal and intact (Figure 2A) compared to the bone density of AIA rats' ankle joints that received no caffeic acid (Figure 2B). Even though the bone density showed major bone loss after the AIA-induction, but the treatment of 125 mg caffeic acid showed remarkable inhibition effect toward the rats' bone loss (Figure 2C).

To further examine the bone loss of AIA rats' ankle joints, densitometry analysis was performed. High rate of bone loss was seen in AIA rats that received no caffeic acid. However, the bone loss rate was reduced after the treatment

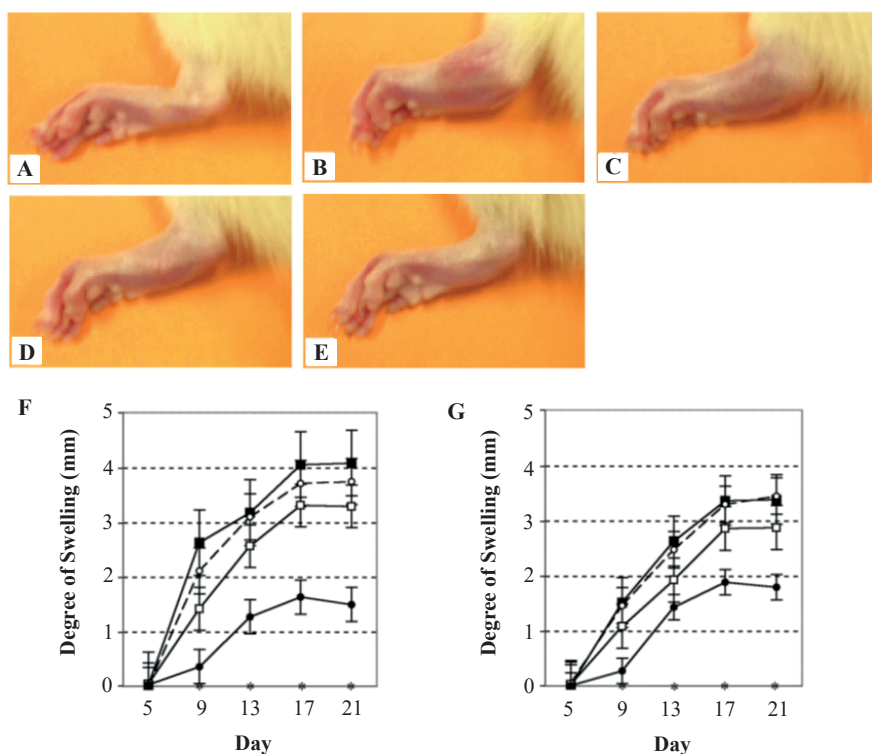


Figure 1. Caffeic acid decreased swelling in ankle joints of AIA rats. A: Ankle joints of rats that were injected with mineral oil (normal); B: Ankle joints of AIA rats that were fed with none; C: Ankle joints of AIA rats that were fed with 5 mg caffeic acid; D: Ankle joints of AIA rats that were fed with 25 mg caffeic acid; E: Ankle joints of AIA rats that were fed with 125 mg caffeic acid; F: Swelling degree of AIA rats' ankle in height; G: Swelling degree of AIA rats' ankle in width (AIA rats fed with none (■), 5 mg (○), 25 mg (□) and 125 mg (●) of caffeic acid). Data represent a typical experiment from 3 independent experiments.

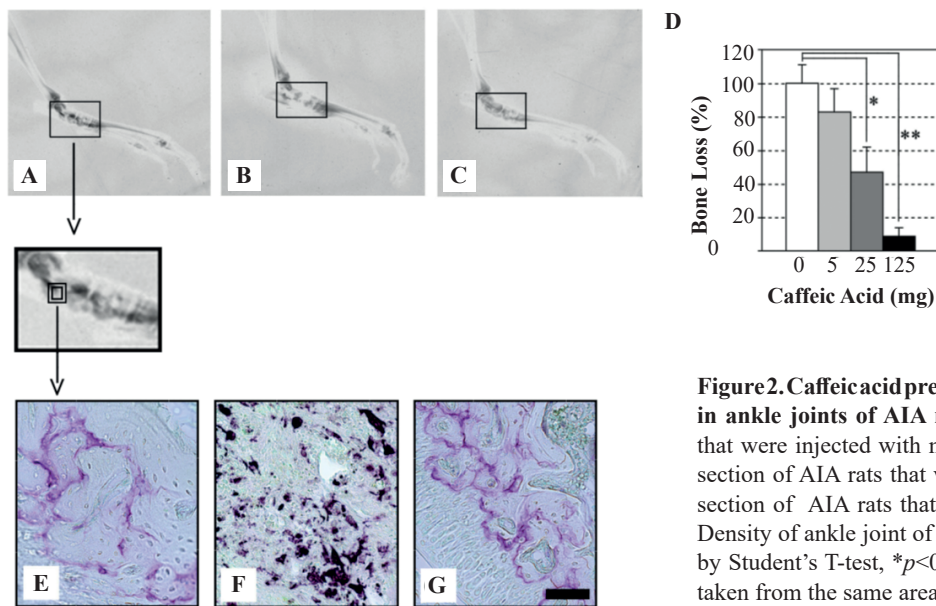


Figure 2. Caffeic acid prevented bone loss and osteoclastogenesis in ankle joints of AIA rats. A&E: Ankle joints section of rats that were injected with mineral oil (normal); B&F: Ankle joints section of AIA rats that were fed with none; C&G: Ankle joints section of AIA rats that were fed with 125 mg caffeic acid; D: Density of ankle joint of all groups were quantified and analyzed by Student's T-test, * $p < 0.05$, ** $p < 0.01$. Figures shown are parts taken from the same area of ankle joints. Data represent a typical experiment from 3 independent experiments. Black bar: 100 μm .

of caffeic acid, especially in 25 mg or higher concentration (Figure 2D). The result of this study showed that bone loss of AIA rats' ankle joints was able to be significantly prevented with the treatments of caffeic acid in the concentration of 25 mg and 125 mg ($p < 0.05$ and $p < 0.01$, respectively).

Caffeic Acid Inhibited Osteoclastogenesis in Ankle Joints of AIA Rats

The osteoclastogenesis was measured by the number of TRAP-positive MNCs in the amputated and decalcified ankle joint of AIA rats. The ankle joint of the rats that received mineral oil showed no TRAP-positive MNCs (Figure 2E). The purple colour staining showed normal condition of the tissue. Meanwhile, the dark brown colour showed high number of TRAP-positive MNCs (Figure 2F), indicating that the osteoclastogenesis in AIA-induced rats' ankle joints was higher than the normal ones. However, low number of TRAP-positive MNCs was observed in ankle joint sections of AIA rats that received 125 mg caffeic acid (Figure 2G), suggesting that the treatment of caffeic acid also inhibited the process of osteoclastogenesis.

Discussion

In previous report, Simon extracts-derived caffeic acid was shown to inhibit osteoclastogenesis in $1\alpha,25(\text{OH})_2\text{D}_3$ -induced rat bone marrow cells and RANKL-TNF α -induced RAW-D cells.(12) Meanwhile, in this study, high degree of swelling and high osteoclastogenesis were found in the

ankle joint of FCA-injected AIA rats, hence caffeic acid was given to reduce the swelling degree and also the osteoclast activity.

Similar to another study (18), in this study the rats that were given mineral oil only showed no swelling ankle. While the administration of FCA has been known to cause some inflammatory reactions (19), including the observed swelling in AIA rat's ankle joint. But a marked inhibition of the swelling was observed in the caffeic acid-treated groups. Moreover, the inhibition was seen to be in a caffeic acid dependent manner. The derivatives of caffeic acid, CAPE, also shown to have the same effect on the swelling of arthritis-induced mice, where the mice treated with CAPE have shown smaller size of swelling compared to the mice that were given none.(20)

Along with that, from radiography and densitometric results, less bone loss was also observed in caffeic acid-treated rats. The higher concentration of caffeic acid administered to the AIA rats, the less bone loss rate was observed. Since it is reported that inhibition of osteoclast differentiation changes a destructive arthritis to a non-destructive form (21), further investigation on osteoclastogenesis showed that FCA-injected AIA rats that were given 125 mg caffeic acid appeared to have less osteoclastogenesis, as comparable as the vehicle (given mineral oil only) rats. This was observed by the less number of TRAP-positive osteoclasts. These results suggested that caffeic acid had potential in inhibiting osteoclastogenesis in ankle joints of AIA rats, so that bone loss could be prevented. This is also in line with previous article that showed the higher concentration of caffeic acid

administration results in higher inhibition rate of TRAP-positive MNCs formation.(12)

Caffeic acid's osteoclastogenesis inhibition potential, might be explain through NF- κ B activity. Caffeic acid concentration especially 10 μ g/mL caffeic acid, which completely inhibited RANKL-TNF- α -induced through NF- κ B luciferase activity.(22) TRAF6, plays a critical role in RANKL-induced osteoclastogenesis. By treatment of caffeic acid, again the significant TRAF6 induced-NF- κ B luciferase activity was diminished, meanwhile no changing of TRAF6's amount was observed.(8) These results suggested that RANKL, TNF- α and TRAF6 played important roles in activating NF- κ B to induce osteoclastogenesis and caffeic acid had a high capability to inhibit this signal pathway.(22) Since this study examined the degree of swallowing and osteoclastogenesis rate after caffeic acid administration, thus to broaden the knowledge, it is also essential to investigate the role of other food compound that might be able to induce similar results with caffeic acid. And since osteoclast activity is under the regulation of RANKL and osteoprotegerin (23), further study to confirm the role of these biomarker in inhibiting osteoclastogenesis is necessary.

Conclusion

The administration of caffeic acid, especially 25 mg and 125 mg, are able to reduce the degree of swallowing, inhibit bone loss, and inhibit osteoclastogenesis in ankle joint of arthritis-induced rats.

Authors Contribution

FS and MIR prepared study concept and design. FS, MIR, and NMD performed processing and acquisition of data. FS and MIR performed analysis and interpretation of results. FS and NMD prepared the draft of the manuscript. MIR and TK made critical revisions of the manuscript. MIR and NMD assisted in administrative, technical, and material support. FS and TK performed supervision of the study. All authors read and approved the final manuscript.

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