RESEARCH ARTICLE

Cafeic Acid Inhibits NFkB Activation of Osteoclastogenesis Signaling Pathway

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Abstract

BACKGROUND: Caffeic acid (3,4-dihydroxy-cinnamic acids) is involved in various green plants. Based on our previous report, a major component of sweet potato extracts, possibly caffeic acid, was shown as a promising inhibitor of osteoclastogenesis. However, the effect of caffeic acid in inhibiting osteoclastogenesis needs to be confirmed. The underlying mechanism needs to be disclosed as well.

METHODS: Caffeic acid in various concentrations was added to *in vitro* osteoclastogenesis of receptor activator nuclear factor kB ligand (RANKL)-tumor necrosis factor alpha (TNFα)-macrophage colony stimulating factor (M-CSF)-induced bone marrow-derived monocyte/macrophage precursor cells (BMMs) and RANKL-TNFα-induced RAW264 cells D-Clone (RAW-D cells). Tartrate resistant acid phosphatase (TRAP) staining was performed and TRAP-positive polynucleated cells (PNCs) were counted. For apoptosis analysis, caffeic acid-treated BMMs, RAW-D cells and osteoclast-like PNCs were subjected to Sub-G1 Apoptosis and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays. To measure NFkB activity, RAW-D cells were transfected with pNFkB-TA-Luc and subjected to Dual Luciferase Reporter Assay System.

RESULTS: Caffeic acid inhibited osteoclastogenesis of RANKL-TNFα-M-CSF-induced BMMs as well as RANKL-TNFα-induced RAW-D cells in a dose dependent manner. Caffeic acid did not induce apoptosis in BMMs, RAW-D cells and osteoclast-like PNCs. RANKL-TNFα-induced NFkB activity in RAW-D was diminished by caffeic acid in a dose dependent manner. Significant NFkB activity inhibition was observed starting from 1 μg/mL caffeic acid.

CONCLUSIONS: Caffeic acid could be a potent osteoclastogenesis inhibitor through inhibition of NFkB activity. Our present study should be further followed up to disclose caffeic acid's possible overlying signaling pathways in inhibiting osteoclastogenesis.

KEYWORDS: caffeic acid, osteoclastogenesis, NFkB, RANKL, TNFα

Indones Biomed J 2011 ; 3 (3) ; 216–222

Introduction

Osteoclasts are specialized cells resorbing bone, which are derived from hematopoietic stem cells. Bone homeostasis is maintained by a constant supply of osteoclasts induced by local factors secreted from osteoblasts (1-4). RANKL and M-CSF are believed to be the most important cytokines for normal supply of osteoclasts. Osteoclast is a principal cell responsible for bone loss in many types of metabolic bone diseases as well as in inflammatory

bone resorption (5). Many efforts have been reported in respect to the regulation of osteoclastogenesis, such as by use of osteoprotegerin (OPG), a decoy receptor for RANKL (2), calcitonin (6), estrogen derivatives (7) and biphosphonates (8). As long-term side effects of these regulators are not fully understood, many attempts have been made to develop a natural agent that have potent activity in inhibiting osteoclastogenesis.

Caffeic acid, a major representative of hydroxy-cinnamic acids, is found in various green plants and is involved in food mainly as an ester with quinic acid, which is designated as chlorogenic acid (3-O-caffeoylquinic acid) (9). Among polyphenols, caffeic acid has the highest absorption through the gut barrier, which reaches 95% of intestinal absorption (10). Caffeic acid is a natural resource with many useful functions in medicine, such as inhibiting N-nitrosation reaction *in vitro*, so that can promote inhibition in formation of mutagenic and carcinogenic N-nitroso compounds (11). One of its derivatives, caffeic acid phenyl ester (CAPE), was also known to have potential to inhibit activation of NF-kB (12,13).

Recently we have shown that a major component of sweet potato extracts, possibly caffeic acid, inhibited osteoclastogenesis (14). In the present study, in order to confirm the effect of caffeic acid and to disclose the mechanism, we investigated the effect of synthetic high-purified caffeic acid on BMMs and RAW-D cells in differentiating into osteoclasts and the possible signaling pathways.

Methods

Cell Culture

RAW-D cells, high RANKL responsive osteoclast precursor cell line clone (15), were cultured in α-MEM (GIBCO-BRL, Grand Island, NY, USA) with 10% FBS (Biosource, Camarillo, CA, USA).

In Vitro Osteoclastogenesis

In vitro osteoclastogenesis in bone marrow cells was done as described by Takayanagi *et al.* (16) with slight modification. Briefly, bone marrow cells were carried out from tibiae and femurs of ddY, specific pathogen free, male, 6 weeks mice. Two million cells per well in a 24-well plate were cultured in α-MEM with 15% FBS containing 10 ng/ml M-CSF (PeproTech, London, UK). After 2 days, adherent cells were used as BMMs after washing out the nonadherent cells including lymphocytes. BMMs were further cultured in the presence of 20 ng/ml RANKL (PeproTech), 1 ng/ml TNFα (Roche Molecular Biochemicals, Mannheim, Germany) and 10 ng/ml M-CSF to generate osteoclast. At the same time, 0-10 μg/ml Caffeic acid (Wako, Osaka, Japan) was added. For *in vitro* osteoclastogenesis in RAW-D cells, 6×10^3 cells per well in a 96-well plate were cultured in α-MEM with 10% FBS containing 20 ng/ml RANKL and 1 ng/ml TNFα. Similarly, 0-10 μg/ml caffeic acid was added at the same time with RANKL and TNFα. Three days later, TRAP staining using Leukocyte Acid Phosphatase Kit (Sigma-Aldrich, St.Louis, MO, USA) was performed and TRAP-positive PNCs (>3 nuclei) were counted. All animal experiments were performed based on the guiding principles of the "Care and Use of Animals" of Kyushu University.

Sub-G1 Apoptosis Assay

Flow cytometry detection of apoptotic cells was performed as described by Sandra *et al.* (17). Briefly, 0-10 μg/ml caffeic acid-treated BMMs or RAW-D cells were harvested and suspended in 1 ml of hypotonic fluorochrome solution (50 μg/ml propidium iodide in 0.1% sodium citrate plus 0.1% Triton X-100). Cell suspensions were placed at 4°C in the dark for 2 hr before the flow cytometric analysis. The propidium iodide fluorescence of individual nuclei was measured with a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA).

TUNEL

This experiment was carried out using Apoptag Peroxidase In Situ Apoptosis Detection Kit (Chemicon, Temecula, CA, USA). Cells were fixed with 1% paraformaldehyde in PBS (pH 7.4) for 10 min at room temperature (RT), washed with PBS 2×5 min, and incubated in pre-cooled ethanol:acetic acid (2:1). Fixed cells were quenched in 3% hydrogen peroxide in PBS for 5 min at RT and rinsed with PBS 2 × 5 min. Later, equilibration buffer was applied on the cells for 10 sec at RT, followed by incubation of TdT enzyme in a humidified chamber at 37°C for 1 hr. To stop the reaction, cells were washed with stop/wash buffer for 10 min at RT and PBS 3×1 min. Anti-digoxigenin peroxidase conjugate was applied on the cells in a humidified chamber for 30 min at RT. For visualization, peroxidase substrate was applied and color development was monitored under the microscope. Cells were later counterstained in 0.5% methyl green for 10 min at RT. Negative control was done by replacing TdT enzyme with PBS, while positive control was provided along with the kit.

NFκB Activity Luciferase Assay

RAW-D cells were transfected with pNFκB-TA-Luc (Clontech, Palo Alto, CA, USA) in a serum free α-MEM using FuGene6 (Roche Molecular Biochemicals). For internal control, pRLTK (an internal control plasmid containing the herpes simplex thymidine kinase promoter linked to a constitutively active *Renilla* luciferase reporter gene) was also transfected. After transfection, cells were treated with 20 ng/ml RANKL, 1 ng/ml TNFα and 0-10 μg/ml caffeic acid. Luciferase assay was performed using the Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA). Briefly, after rinse with PBS, 1X passive lysis buffer (PLB) was added to the cells in the amount as indicated in the protocol. Cells were rocked for 15 min at RT. Then, 20 μl of each cell lysate was immediately applied to 96-well plate for measuring NFkB luciferase activity using TR717 Microplate Luminometer (Tropix, Bedford, MA, USA).

Statistical Analysis

Results were expressed as the mean ± SEM. Analyses were 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. A probability value <0.05 was considered to be statistically significant. Analyses were done on each experiment carried out at least three times.

Results

Inhibition of osteoclastogenesis by caffeic acid

Under stimulation of 20 ng/ml RANKL, 1 ng/ml TNFα and 10 ng/ml M-CSF, BMMs were successfully differentiated into osteoclasts (Figure 1A). A high number of TRAP-positive PNCs was observed in the sample without addition of caffeic acid, in contrast, a low number of TRAP-positive PNCs was seen when cultures were treated with caffeic acid. The caffeic acid osteoclastogenesis inhibition was shown in a dose dependent manner (Figure 1B-E). Significant osteoclastogenesis inhibition was observed starting from 0.1 μg/ml caffeic acid.

Similar inhibition activity of caffeic acid was seen in RAW-D cells (Figure 2). Without addition of caffeic acid, RAW-D cells that were treated with 20 ng/ml RANKL and 1 ng/ml TNFα (the optimal combination of RANKL and TNFα to induce RAW-D cells to form osteoclast-like PNCs (15,18)), were observed to have a high number of TRAP-positive PNCs (Fig. 2A). By addition of caffeic acid, less number of TRAP-positive PNCs was observed. The potential of caffeic acid in inhibiting RANKL-TNFα-stimulated osteoclastogenesis in RAW-D cells was also dependent on its concentration (Fig. 2B-E). Significant osteoclastogenesis inhibition was observed starting from 1 μg/ml caffeic acid.

Caffeic acid did not cause apoptosis in BMMs, RAW-D cells and PNCs

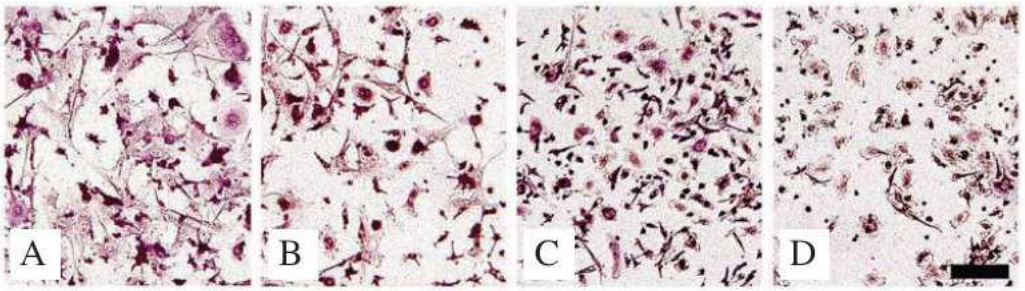
BMMs and RAW-D cells were treated with 0-10 μg/ml caffeic acid. As shown in Figure 3A, 0.1-10 μg/ml caffeic acid-treated BMMs did not demonstrate a marked increase in apoptosis as compared with the untreated control (0 μg/ml caffeic acid. Similar results were also seen in RAW-D cells treated with 0.1-10 μg/ml caffeic acid (Figure 3B). Additionally, TUNEL results also confirmed that there was no distinct difference in the number of TUNEL-positive cells between 0 (C) and 10 (D) μg/ml caffeic acid-treated RAW-D cells. To test whether caffeic acid induced apoptosis in osteoclasts-like PNCs, we treated osteoclast-like PNCs (RANKL-TNFα-induced RAW-D cells) with 10 μg/ml caffeic acid (Figure 3E) and TRAP-stained. The result showed that caffeic acid did not decrease the number of TRAP-positive PNCs significantly.

Caffeic acid diminished NFκB activity

Under stimulation of 20 ng/ml RANKL and 1 ng/ml TNFα for 12 hr, NFkB activity of RAW-D cells was significantly elevated 4 folds as compared with the control (0 μg/ml caffeic acid) (Figure 4). Addition of caffeic acid diminished NFkB activity in accordance with its potential to inhibit osteoclastogenesis. NFkB activity inhibition was observed in caffeic acid in a dose dependent manner. Significant NFkB activity inhibition was observed starting from 1 μg/ml caffeic acid.

Discussion

In our recent report, sweet potato extracts were shown to inhibit osteoclastogenesis (14). The extracts were composed of caffeic acid, a component that is possibly an active agent in inhibiting osteoclastogenesis. In our present study, we applied a synthetic high-purified caffeic acid, from which we found that caffeic acid inhibited osteoclastogenesis in mouse BMMs stimulated with M-CSF-RANKL-TNFα and in RAW-D cells stimulated with RANKL-TNFα. Hence, the potential of caffeic acid in inhibiting osteoclastogenesis was confirmed.



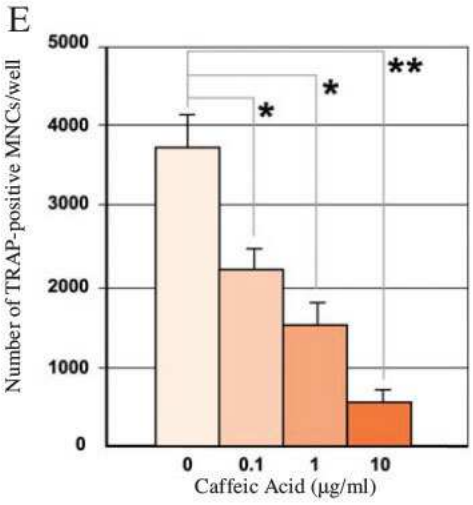
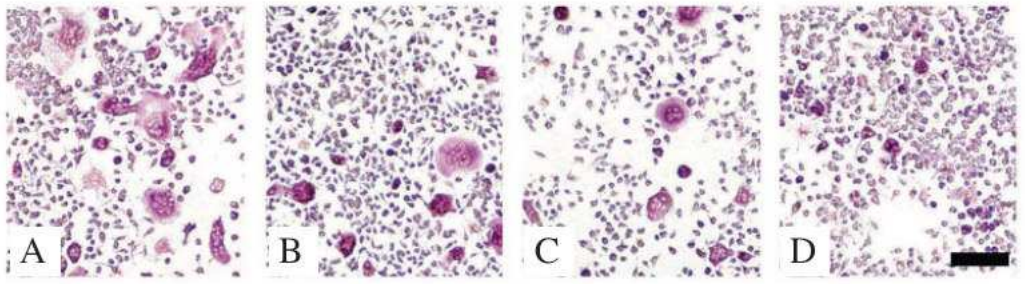


Figure 1. Caffeic acid inhibited RANKL-TNFα-M-CSF-induced osteoclastogenesis in BMMs. BMMs were isolated according to Materials and Methods. BMMs were cultured in the presence of 20 ng/ml RANKL, 1 ng/ml TNFα, 10 ng/ml M-CSF and 0 (A), 0.1 (B), 1 (C) or 10 (D) µg/ml caffeic acid for 3 days. Cells were fixed and TRAP-stained. E: TRAP-positive PNCs were counted and analyzed by Student's t-test, *p<0.05, **p<0.01. Data represent a typical experiment from 3 independent experiments. Bar: 100 µm.



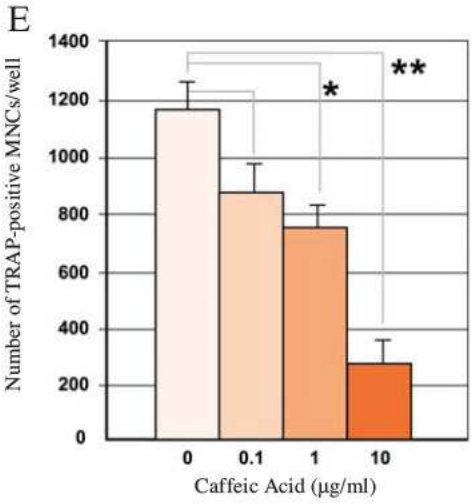


Figure 2. Caffeic acid inhibited RANKL-TNFα-induced osteoclastogenesis in RAW-D cells. Six thousand RAW-D cells were cultured in the presence of 20 ng/ml RANKL, 1 ng/ml TNFα and 0 (A), 0.1 (B), 1 (C) or 10 (D) µg/ml caffeic acid for 3 days. Cells were fixed and and TRAP-stained. E: TRAP-positive PNCs were counted and analyzed by Student's t-test, *p<0.05, **p<0.01. Data represent a typical experiment from 3 independent experiments. Bar: 100 µm.

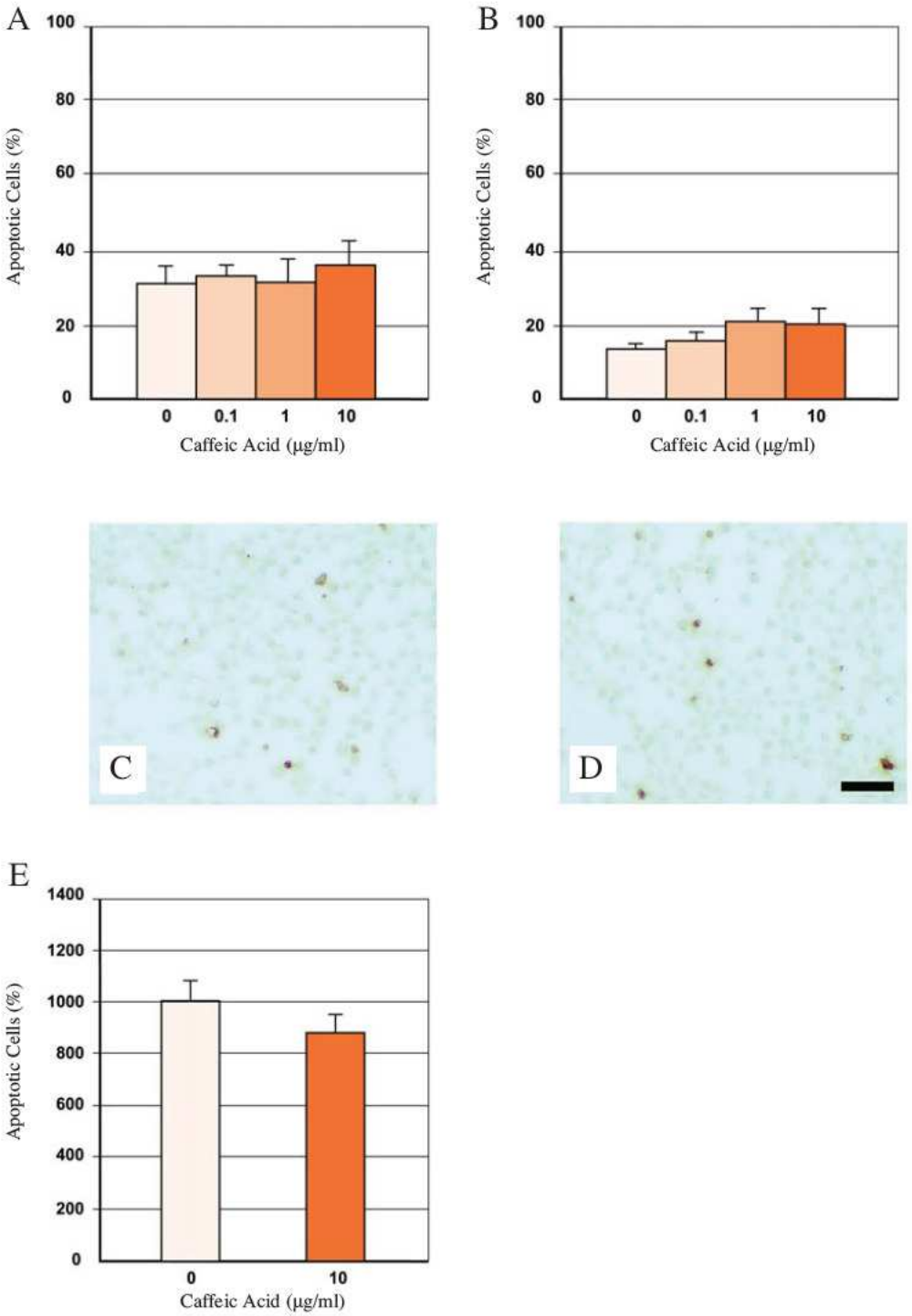


Figure 3. Caffeic acid did not induce apoptosis in BMMs, RAW-D cells and PNCs. Different concentrations of caffeic acid as indicated in the panel were added to $2x10^5$ BMMs (A) and RAW-D cells (B). After 3 days, cells were collected and subjected to Sub-G1 Apoptosis assay. For confirmation, TUNEL expression of 0 (C) and 10 (D) µg/ml caffeic acid-treated RAW-D cells for 3 days was demonstrated. E: PNCs derived from 20 ng/ml RANKL-1 ng/ml TNFα−treated RAW-D cells for 3 days, were added with 10 µg/ml caffeic acids for 2 days. Cells were fixed, TRAP-stained and counted. All of these experiments were repeated 3 times. Bar: 100 µm.

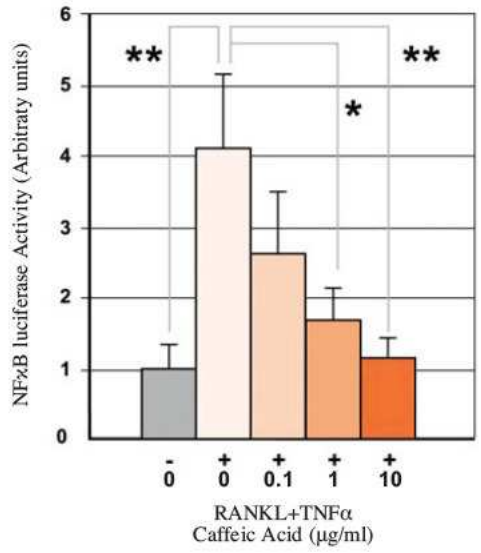


Figure 4. Caffeic acid inhibited RANKL-TNFα-induced NFκB luciferase activity in RAW-D cells. One hundred thousand RAW-D cells were transfected with pNFκB-TA-Luc. After 30 hr, transfected cells were treated with 20 ng/ml RANKL, 1 ng/ml TNFα and 0-10 μg/ml caffeic acid as indicated in the panel for 12 hr. Cells were lysed and subjected to NFκB Luciferase assay. Data were analyzed by Student's t-test, *p<0.05, **p<0.01. Data represent a typical experiment from 3 independent experiments.

We suspect there is a possibility of caffeic acid being able to induce apoptosis in BMMs and RAW-D cells, which might distort the idea of caffeic acid's function in osteoclastogenesis inhibition. In the present results, we clarified that caffeic acid did not induce apoptosis in BMMs, RAW-D cells and osteoclast-like PNCs. These results suggested that caffeic acid merely affects differentiation process not by inducing apoptosis of progenitor cells or mature osteoclasts.

NFkB has been known as an important transcription factor in osteoclastogenesis, therefore, selective inhibition of NFkB blocks osteoclastogenesis and prevents inflammatory bone destruction *in vivo* (19). CAPE, a derivative of caffeic acid was shown to inhibit activation of NFkB in cancer cell model (12, 13). To understand the mechanism of caffeic acid's osteoclastogenesis inhibition potential, we investigated the activation of NFkB as well (19). In our present study, we found that caffeic acid inhibited RANKL-TNFα-induced NFkB activity in RAW-D cells. Taken together, caffeic acid could be a potent osteoclastogenesis inhibitor through inhibition of NFkB activity. We suggest that our present study should be followed up by further studies to disclose caffeic acid's possible overlying signaling pathways in inhibiting osteoclastogenesis.

Acknowledgements:

This project was supported by Grant-in-Aid for Scientific Research from JSPS (No. P 03145). The authors are thankful to Drs. Noboru Yamaguchi and José Guillermo Martinez for their technical supports.

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