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ORIGINAL PAPER

Homeopathic *Rhus toxicodendron* has dual effects on the inflammatory response in the mouse preosteoblastic cell line MC3T3-e1

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Background: **Homeopathic remedy *Rhus toxicodendron* (*Rhus tox*) is used for several symptoms including skin irritations, rheumatic pains, mucous membrane afflictions, and typhoid type fever. Previously, we reported that *Rhus tox* treatment increased the cyclooxygenase-2 (*COX-2*) mRNA expression in primary cultured mouse chondrocytes.**
Methods: **A preosteoblastic mouse cell line, MC3T3-e1, was treated with different homeopathic dilutions of *Rhus tox* and the *COX-2* mRNA and protein expression was examined using reverse transcriptase-polymerase chain reaction (RT-PCR) and immunoblotting. Additionally, nitric oxide (NO) generation was examined in LPS-induced MC3T3-e1 cells using a Griess reaction assay.**
Results: **Stimulation with different concentrations of *Rhus tox* increased the expression of *Cox2* mRNA, with 30X *Rhus tox* showing the most prominent increase in mRNA expression. In addition, treatment with 30X *Rhus tox* significantly increased prostaglandin E2 (PGE2) release compared with other homeopathic dilutions. However, the COX-2 protein expression level differed slightly from its mRNA expression, because the 30C *Rhus tox* treatment increased COX-2 protein to a greater extent compared with other dilutions. NO generation was dramatically decreased in MC3T3-e1 cells after *Rhus tox* treatment co-stimulated with lipopolysaccharide.**
Conclusion: **Homeopathic dilution of *Rhus tox* has a dual activity that increases COX-2 expression and decreases NO generation, thus modulating inflammation. Further study is needed to examine the cellular signaling mechanisms that are associated with inflammatory regulation by *Rhus tox* treatment in greater detail.** *Homeopathy* (2016) **105**, 42–47.

Keywords: *Rhus toxicodendron* (*Rhus tox*); Cyclooxygenase-2 (COX-2); Prostaglandin E2 (PGE2); Nitric oxide (NO) generation

Introduction

Arthritis is a joint disorder that involves inflammation of the joints characterized by pain, swelling and stiffness that results from infection, trauma, degenerative changes, metabolic disturbances or other causes. Inflammatory arthritis includes rheumatoid arthritis, psoriatic arthropathy, inflammatory bowel disease, juvenile idiopathic arthritis, and systemic lupus erythematosus.[1] There are many factors involved in the inflammation mechanism including cyclooxygenase-2 (COX-2), prostaglandins, nitric oxides (NOs), and many cytokines. The treatment of inflammatory arthritis is intended to reduce or minimize the associated pain and inflammation using steroids and non-steroidal anti-inflammatory drugs as well as to decelerate the

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progress of disease by using disease-modifying anti-rheumatic drugs. Arthritic patients tend to search for alternative treatments that are effective and less toxic than pharmaceutical pills.[2,3]

The homeopathic dilution of *Rhus toxicodendron* (*Rhus tox*) has been used for the treatment of inflammatory conditions including skin eruptions, back pains and stiffness, irritability and restlessness, and rheumatoid arthritis.[4–6] Many studies have reported that *Rhus tox* showed immunomodulatory effects in experimental animal models.[6–8] The activity of homeopathic dilutions of *Rhus tox* has primarily been demonstrated through clinical trials using animal models. Previously, we examined the expression of COX-2 and prostaglandin E2 (PGE2) in *Rhus tox*-stimulated primary cultured mouse chondrocytes using reverse transcriptase-polymerase chain reaction (RT-PCR), quantitative (or real-time) RT-PCR (qRT-PCR), and immunoblotting.[9]

COX is composed from two isoforms, COX-1 and COX-2.[10] COX-1 is constitutively expressed in most tissues.[10] By contrast, COX-2 expression is induced by inflammation and is primarily responsible for the synthesis of the prostanoids (prostaglandins and thromboxanes) involved in pathological processes.[10–12] COX-2 promotes the release of the pro-inflammatory mediator PGE2, and COX-2 inhibitors suppress PGE2 production.[13] The induction of COX-2 expression is closely associated with the release of PGE2, and treatment with a COX-2 inhibitor reduces inflammation in animal models.[11] COX-2 and PGE2 are constitutively active or inducible through inflammatory stimuli and are important in normal joint pathophysiology. PGE2 is a principal mediator of the inflammatory response.[14,15]

NO is a multifunctional signaling molecule that regulates various cellular events in inflammation. NO stimulates an anti-inflammatory effect under normal physiological conditions, but is also considered to be a pro-inflammatory mediator because of its overproduction in inflammatory situations.[16] NO is synthesized from L-arginine by NO synthase (NOS). NOS activity depends upon three distinct isoforms depending on tissue type. Two of the isoforms are constitutively expressed, one primarily expressed in endothelial cells (eNOS) and the other in neuronal cells (nNOS). The third family member is inducible NOS (iNOS), which is induced by inflammatory cytokines.[17–19] Two members, iNOS and nNOS, are soluble and found predominantly in the cytosol; whereas, eNOS is membrane-associated.

This study investigated the effects of *Rhus tox* in inflammatory modulation using a mouse preosteoblastic cell line, and examined COX-2 gene expression and NO production. Stimulation of MC3T3-e1 cells by *Rhus tox* increased *Cox2* mRNA and protein, elevated PGE2 protein production, and decreased NO production. From these results, we propose that homeopathic dilutions of *Rhus tox* have dual activities in inflammation modulation in this mouse preosteoblastic cell line.

Materials and methods

Preparation of reagents

The liquid dilutions of *Rhus tox* at 4X, 30X, 30C, and 200C were purchased from Boiron (Newtown Square, PA) and used to prepare 1:10 dilutions in cell culture media according to the manufacturer's instructions. The liquid form of *Rhus tox* was supplied in 20% ethanol (EtOH), and we prepared a 20% (v/v) EtOH solution for use in the 1:10 dilution. A final concentration of 2% (v/v) EtOH or 2% (v/v) *Rhus tox* solution in the cell culture media was used in the control and test group. Unless otherwise mentioned, all chemicals were purchased from Sigma−Aldrich (St. Louis, MO).

Culture of MC3T3-e1 cells

MC3T3-e1 subclone 4 cells (American Type Culture Collection [ATCC], CRL-2593, Manassas, VA) were grown in alpha minimum essential medium (GIBCO, A1049001, Grand Island, NY) supplemented with 10% (v/v) fetal bovine serum (FBS, GIBCO), and 100 units/100 μg/ml penicillin-streptomycin (GIBCO). Unless specified otherwise, MC3T3-e1 cells were cultured at 37°C in a humidified 5% CO_2 atmosphere.

Cell proliferation assay

Cell proliferation was measured using a CellTiter 96 non-radioactive cell proliferation assay kit to measure changes in absorbance at a specific wavelength using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) according to the manufacturer's instructions (Promega, Madison, WI). Briefly, the MC3T3-e1 cells were plated onto 96-well plates at a density of 1.0×10^4 cells/well and were cultured for up to 24 h. Dilutions (4X, 30X, 30C, or 200C) of the homeopathy remedy *Rhus tox* or 2% EtOH, as a control, were added to the cells and co-cultured for 48 h at 37°C in a humidified 5% CO_2 atmosphere. MTT-phenazine methosulfate solution (15 μl/well) was added, and the cells were incubated for 4 h at 37°C in a humidified 5% CO_2 atmosphere. Subsequently, the reaction was terminated upon the addition of 100 μl of Solubilization Solution/Stop Mix. The absorbance was measured at 570 nm using a microplate reader (BioTek Instruments, Winooski, VT). The data represent the average of three wells in one independent experiment repeated four times.

RT-PCR

To examine *Cox2* expression under treatment with various concentrations of *Rhus tox*, *Cox2* mRNA expression was analyzed using RT-PCR. MC3T3-e1 cells were grown in media containing 4X, 30X, 30C, or 200C homeopathic dilutions of *Rhus tox* or 2% EtOH for 48 h, and total RNA was extracted using an RNeasy kit (Qiagen, Austin, TX) according to the manufacturer's instructions. After extraction, the RNA was converted to cDNA through reverse transcription. Reverse transcription was performed

using 1 μg of total RNA with TOPscript RT DryMIX (Enzynomics, Seoul, Korea). The PCR reactions were performed using a GeneAmp PCR System 9700 (Applied Biosystems, Carlsbad, CA) with Ampli*Taq* DNA polymerase (PE Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The following primers (Macrogen, Seoul, Korea) were used for the RT-PCR reactions: glyceraldehyde phosphate dehydrogenase (*Gapdh*, 587 bp): sense 5′-TCACGCCACCCAGAAGAC-3′, antisense 5′-TCACTGCCACCCAGAAGAC-3′; *Cox2*: sense 5′-GGTCTGGTGCCTGGTCTGATGAT-3′, antisense 5′-GTCCTTTCAAGGAGAATGGTGC-3′. The PCR conditions included denaturation (95°C, 3 min), then amplification and quantification (95°C, 20 s; 62°C, 10 s; 72°C 30 s; 22 cycles for *Gapdh* and 95°C, 20 s; 63°C, 10 s; 72°C 30 s; 28 cycles for *Cox2*), followed by a final elongation (72°C for 5 min). The amplified PCR products were visualized on 1.5% agarose gels.

Immunoblot analysis

MC3T3-e1 cells were stimulated with 2% EtOH or 4X, 30X, 30C, or 200C homeopathic dilutions of *Rhus tox* for 48 h and lysed in lysis buffer (1% Triton X-100, 50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 10 mM NaF, 1 mM pyrophosphate, and 2 mM Na_3VO_4) supplemented with a protease inhibitor cocktail (Sigma−Aldrich) on ice for 10 min. The protein concentration was determined using the Bradford method (Bio-Rad Laboratories, Hercules, CA), and proteins were separated using SDS-PAGE. The immunoblot analysis was performed using anti-COX-2 antibody (R&D Systems, Minneapolis, MN) and reprobed using anti-actin antibody (EMD Millipore, Billerica, MA). The bound antibodies were detected through enhanced chemiluminescence (Pierce Chemical, Rockford, IL) using a LAS 4000 mini biomolecular imager (GE Healthcare, Uppsala, Sweden).

PGE2 assay

To examine PGE2 expression in MC3T3-e1 cells, a PGE2 assay kit was used according to the manufacturer's instructions (R&D Systems). MC3T3-e1 cells were stimulated with 2% EtOH or 4X, 30X, 30C, or 200C homeopathic dilutions of *Rhus tox* for 48 h, and the culture supernatants were used for the assay.

NO generation assay

The analysis of NO was accomplished by measuring the amount of nitrate in the cell culture supernatant using the Griess assay.[20] Briefly, MC3T3-e1 cells (1.0×10^4 cells/ml) were cultured on 96-well plates for 24 h, then stimulated with or without lipopolysaccharide (LPS) for 24 h in culture, and co-cultured with various concentrations of *Rhus tox* for 48 h. LPS is a bacterial endotoxin and broadly used to induce inflammation in both *in vivo* and *in vitro* experiments.[21] The culture supernatants were reacted with the Griess reagent (Sigma−Aldrich), and the absorbance at 550 nm was measured to determine NO production using a serial dilution of $NaNO_2$ as a standard curve. The data represent the average of duplicates of one independent experiment that was repeated three times.

Statistical analysis

For statistical comparison, the experiments were repeated three times. The values are presented as the mean ± SEM (standard error of the means). An independent sample-test and analysis of variance were used to evaluate the data with SigmaPlot 10.0 (Systat Software Inc., San Jose, CA) and $p < 0.05$ was considered statistically significant.

Results

Cell proliferation was not altered by *Rhus tox* treatment

The possibility that there might be cytotoxic effects of *Rhus tox* treatment on primary cultured mouse MC3T3-e1 cells was evaluated using an MTT colorimetric assay.[22] Briefly, the cells were stimulated with 2% EtOH or 4X, 30X, 30C or 200C homeopathic dilutions of *Rhus tox* for 48 h, and the cell proliferation was analyzed (Figure 1). Stimulation with a homeopathic dilution of *Rhus tox* did not affect the survival and proliferation of MC3T3-e1 cells.

Cox2 mRNA expression was elevated in *Rhus tox*-stimulated MC3T3-e1 cells

To investigate the direct effects of diluted *Rhus tox* MC3T3-e1 cells were stimulated with 2% EtOH, as a control, or 4X, 30X, 30C or 200C homeopathic dilutions of *Rhus tox* and co-cultured for 48 h. The expression of *Cox2* mRNA was analyzed using RT-PCR. *Cox2* expression was most increased in the 30X *Rhus tox*-stimulated MC3T3-e1 cells compared with the other treatments (Figure 2).

Elevated PGE2 release in *Rhus tox*-stimulated MC3T3-e1 cells

To examine the potential correlation of PGE2 release with increased COX-2 activity MC3T3-e1 cells were stimulated with homeopathic dilutions of *Rhus tox*, and PGE2 production was examined. The 30X *Rhus tox*-stimulated MC3T3-e1 cells showed higher levels of PGE2 production than the other homeopathic dilutions and the control (Figure 3).

Increased expression of COX-2 protein in *Rhus tox*-stimulated MC3T3-e1 cells

COX-2 protein expression in *Rhus tox*-treated cells was examined by immunoblotting analysis. As shown in Figure 4, 30C *Rhus tox*-stimulated MC3T3-e1 cells showed the highest expression levels of COX-2. This result differed slightly from the *COX-2* mRNA expression that peaked at 30X, but showed that *Rhus tox* treatment consistently up-regulated COX-2 protein expression. Based on these results, we conclude that treatment with a homeopathic dilution of *Rhus tox* is directly associated with the COX-2 gene expression and the inflammatory response in MC3T3-e1 cells.

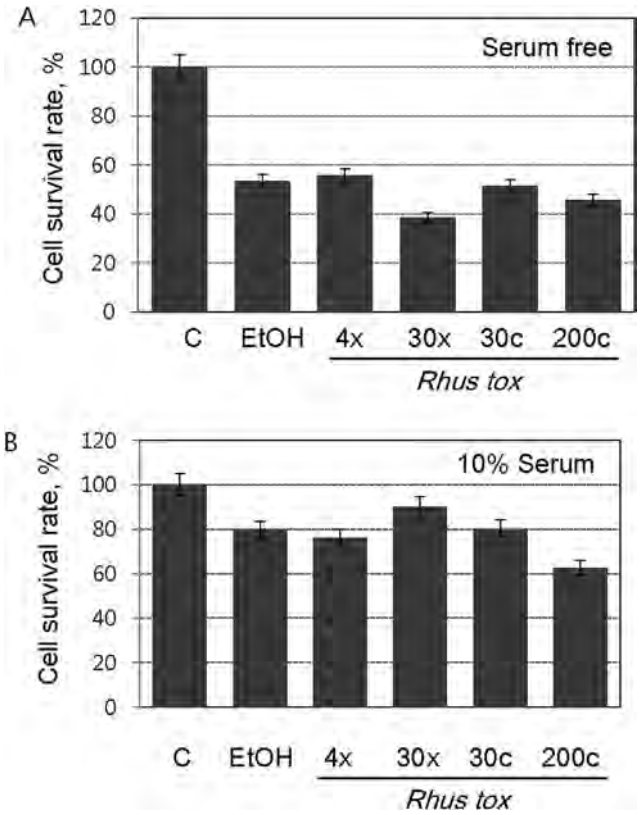

Figure 1 Cell proliferation of MC3T3-e1 cells was unaltered after treatment with a homeopathic dilution of *Rhus tox*. Cell survival rates are calculated using Absorbance 570 nm $\left(\text{survival rate } (\%) = \frac{\text{Abosrbance of sample}}{\text{Asorbance of control}} \times 100\right)$. The chart represents the average levels (±SEM; $p < 0.05$). C: untreated MC3T3-e1 cells. A: without 10% FBS, B: with 10% FBS.

Rhus tox reduced NO generation

To determine the effect of *Rhus tox* on NO generation, MC3T3-e1 cells were activated with or without LPS for 24 h. After an optimized LPS concentration (1 μg/ml LPS) was determined (data not shown), MC3T3-e1 cells

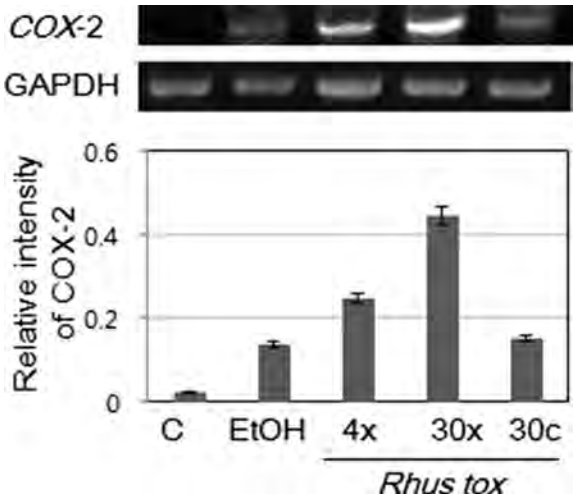

Figure 2 *Cox2* mRNA expression was maximally increased in cells treated with a 30X homeopathic dilution of *Rhus tox*. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as a loading standard. C: untreated MC3T3-e1 cells.

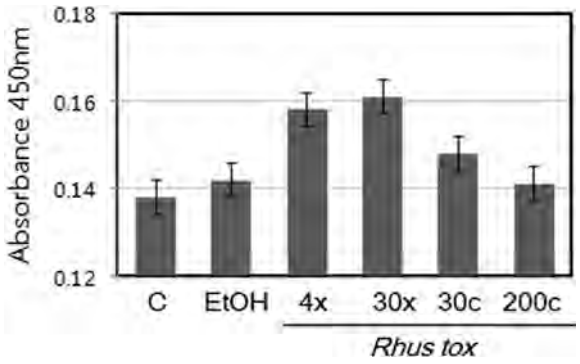

Figure 3 PGE2 expression was increased after treatment with 4X and 30X homeopathic dilutions of *Rhus tox*. Stimulation with 30X homeopathic diluted *Rhus tox* induced higher PGE2 expression in preosteoblastic cells compared with other treatments. The chart represents the average levels (±SEM; $p < 0.05$). C: untreated MC3T3-e1 cells.

were activated for 24 h and co-cultured with various concentrations of *Rhus tox*. As shown in Figure 5, LPS-stimulated MC3T3-e1 cells had decreased NO generation in response to *Rhus tox* treatment.

Discussion

Many basic science studies and clinical investigations to date have evaluated the anti-inflammatory and anti-arthritis activity of natural plants such as *Eugenia jambos*, *Phyllanthus amarus*, *Nigella dativa* L., and others.[3,23–25] Moreover, the homeopathic remedy *Rhus tox* has been used to treat joint pain with stiffness and typically causes muscular, articular, and ligament pain and induces dermatitis.[8] *Rhus tox* modulates arthritis[6, 26] and exhibits anti-inflammatory and proinflammatory effects in Carrageenan-induced paw edema in rats.[8,27] These studies were mainly accomplished using animal models. Patel et al. demonstrated that homeopathic *Rhus tox* possesses immunomodulatory activity in human polymorphonuclear cells.[7] de Oliveira et al. investigated that the action of highly diluted substances and tinctures

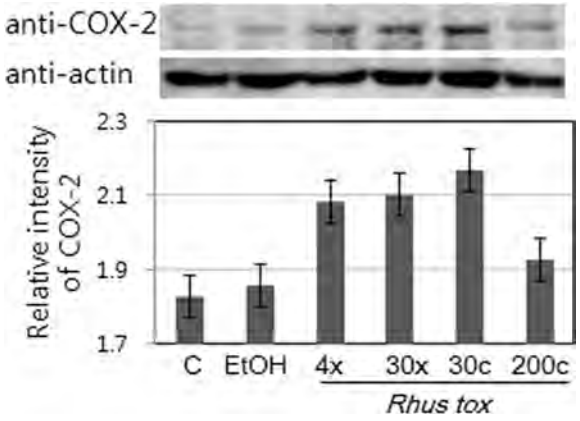

Figure 4 MC3T3-e1 cells stimulated with a 30C homeopathic dilution of *Rhus tox* showed higher COX-2 protein induction compared with other treatments. Actin was used as a loading control. C: untreated MC3T3-e1 cells.

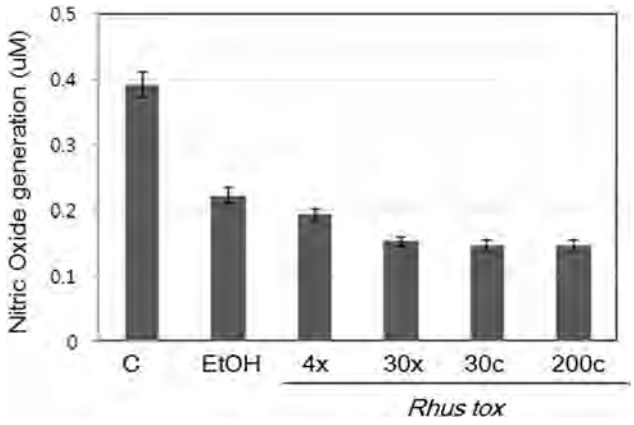

Figure 5 LPS-induced MC3T3-e1 cells treated with homeopathic dilutions of *Rhus tox* displayed reduced NO generation. The chart represents the average levels (±SEM; $p < 0.05$).

on cells from the immune system including *Rhus tox*, and showed that highly diluted tinctures modulate the immune response.[28] Although, many studies have reported the anti-inflammatory and anti-arthritis effect of *Rhus tox*, the mechanisms underlying the effects observed in both *in vivo* and *in vitro* systems remains unclear.

This study demonstrates the anti-arthritis activity of homeopathic dilution of *Rhus tox* using a mouse preosteoblastic cell line model. Previously, we reported that *Rhus tox* treatment increased *Cox2* mRNA expression in a primary cultured mouse chondrocytes using advanced methods.[9] In our previous study, *Rhus tox* treatment was associated with the expression of COX-2 and PGE2, thus having anti-inflammatory effects. Although we examined the effect of homeopathic dilution treatment by advanced molecular biological methods in that report, those analyses was limited to using primary cultured mouse cells. The advantage of primary cultures is that the conditions closely resemble the *in vivo* cell phenotype, but these cells have a limited cell passage number. Therefore, consistently investigating and understanding the complex interaction of *Rhus tox* requires an *in vitro* cell system that might be useful for studying the intracellular signaling pathways.

To determine the effects of *Rhus tox* on inflammation in our current study, we examined the primary factors associated with inflammation including COX-2 and PGE2 expression and NO generation. Specifically, we measured *Cox2* mRNA expression in MC3T3-e1 cells stimulated by homeopathically diluted *Rhus tox*. These results clearly correspond with our previous finding that stimulation with *Rhus tox* increases *Cox2* mRNA expression. COX-2 is dramatically up-regulated during inflammation in patients with rheumatoid arthritis,[29] and a COX-2 inhibitor shows anti-inflammatory activity.[29] Previous studies suggest that the expression of COX-2 is regulated through a broad spectrum of mediators involved in inflammation,[10] and PGE2 is also up-regulated in various cell lines.[10,30,31] We also have reported that the *Rhus tox* treatment increased COX-2 and PGE2 production in mouse primary cultured chondrocytes.[9] We see a consistent correlation between COX-2 expression and PGE2 production in mouse chondrocytes and MC3T3-e1 cells in response to *Rhus tox* treatment.

NO is a cellular signaling molecule that plays a key role in inflammation including pro-inflammatory and anti-inflammatory aspects, and has both host-protective and host-damaging actions during infections.[32] NO is generated during immune and inflammatory responses and is associated with inflammatory disease. In our present study, LPS-stimulated MC3T3-e1 cells treated with *Rhus tox* showed reduced NO generation, suggesting that *Rhus tox* treatment reduces inflammation. However, further investigation required to understand the involvement of this treatment in NO signaling pathways in terms of more precise mechanisms.

Collectively, our present study findings shows that the stimulation of mouse MC3T3-e1 cells with *Rhus tox* induces the up-regulation of *COX-2* mRNA and protein, thereby enhancing PGE2 production, whereas, NO generation is reduced. These results suggest that the homeopathic remedy *Rhus tox* modulates inflammation, although the precise mechanism is still unknown. Our results provide *in vitro* evidence for the effectiveness of the homeopathic remedy *Rhus tox* and lay the foundation for further research. Additionally, our current data provide a cellular model for the homeopathic dilution of *Rhus tox*. Future studies will examine the intracellular mechanisms of NO signaling in *Rhus tox*-stimulated MC3T3-el cells and additional animal models.

Conclusions

COX-2 is up-regulated during inflammation and plays a role in PGE2 production. Treatment with a 4X homeopathic dilution of *Rhus tox* increases both COX-2 and PGE2 production, but decreases NO generation in MC3T3-e1 cells. Based on these results, we suggest that *Rhus tox* has a dual activity that modulates inflammatory responses in MC3T3-e1 cells.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

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