Evaluation of the effects of Cimicifugae Rhizoma on the morphology and viability of mesenchymal stem cells

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Abstract. Cimicifugae Rhizoma is a traditional herbal medicine used to treat various diseases in Korea, China and Japan. Cimicifugae Rhizoma is primarily derived from *Cimicifuga heracleifolia* Komarov or *Cimicifuga foetida* Linnaeus. Cimicifugae Rhizoma has been used as an anti-inflammatory, analgesic and antipyretic remedy. The present study was performed to evaluate the extracts of Cimicifugae Rhizoma on the morphology and viability of human stem cells derived from gingiva. Stem cells derived from gingiva were grown in the presence of Cimicifugae Rhizoma at final concentrations that ranged from 0.001 to 1,000 μg/ml. The morphology of the cells was viewed under an inverted microscope and the analysis of cell proliferation was performed using a Cell Counting kit-8 (CCK-8) assay on days 1, 3, 5 and 7. Under an optical microscope, the control cells exhibited a spindle-shaped, fibroblast-like morphology. The shapes of the cells in the groups treated with 0.001, 0.01, 0.1, 1 and 10 μg/ml Cimicifugae Rhizoma were similar to the shapes in the control group. Significant alterations in morphology were noted in the 100 and 1,000 μg/ml groups when compared with the control group. The cells in the 100 and 1,000 μg/ml groups were rounder, and fewer cells were present. The cultures that were grown in the presence of Cimicifugae Rhizoma at a concentration of 0.001 μg/ml on day 1 had an increased CCK-8 value. The cultures grown in the presence of Cimicifugae Rhizoma at a concentration of 10 μg/ml on day 7 had a reduced CCK-8 value. Within the limits of this study, Cimicifugae Rhizoma influenced the viability of stem cells derived from the gingiva, and its direct application onto oral tissues may have adverse effects at high concentrations. The concentration and application time of Cimicifugae Rhizoma should be meticulously controlled to obtain optimal results.

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Introduction

Cimicifugae Rhizoma, also known as Seungma in Korea, Shengma in China and Shoma in Japanese is a traditional herbal medicine that is used to treat various diseases in these countries (1). Cimicifugae Rhizoma is primarily derived from *Cimicifuga heracleifolia* Komarov or *Cimicifuga foetida* Linnaeus (2). Cimicifugae Rhizoma has traditionally been used as an anti-inflammatory, analgesic and antipyretic remedy (3-6). It has been shown to induce alkaline phosphatase synthesis in rat calvarial osteoblasts when tested *in vitro* (7). Cimicifugae Rhizoma has also been suggested to be useful for the treatment of dental diseases, including periodontitis (8). It has demonstrated antimicrobial activity against *Porphyromonas gingivalis*, a common bacterium in oral biofilms, when tested *in vitro* (8), and has also shown chelating ability, which may be applied for the prevention of oral calcium phosphate precipitation (calculus formation) (9).

A limited study has been performed to evaluate the effects of Cimicifugae Rhizoma on cell viability (10). Cimicifugae Rhizoma extract induced G_0/G_1 cell cycle arrest of hepatocellular carcinoma at a low concentration (25 μg/ml), triggered G_2/M arrest and apoptosis of the hepatocellular carcinoma at higher concentrations (50 and 100 μg/ml) and inhibited the growth of implanted mouse tumors in a dose-dependent manner, with a growth inhibitory rate of 63.3% at 200 mg/kg (10). However, limited information is currently available regarding the effects of Cimicifugae Rhizoma on dental tissue, including mesenchymal stem cells derived from gingiva.

The aim of the present study was to evaluate the effects of extracts of Cimicifugae Rhizoma on the morphology and viability of human stem cells derived from gingiva. To the best of our knowledge, this study is the first to elucidate the effect of Cimicifugae Rhizoma on stem cells derived from gingiva.

Materials and methods

Preparation of materials. The dry roots of *Cimicifuga heracleifolia* Komarov (500 g; Chungju Hospital of Korean Medicine, College of Korean Medicine, Semyung University, Chungju, Republic of Korea) were immersed in distilled water and boiled under reflux for 2 h 30 min, and the resulting extract was centrifuged at 5,000 x g for 10 min. The supernatant

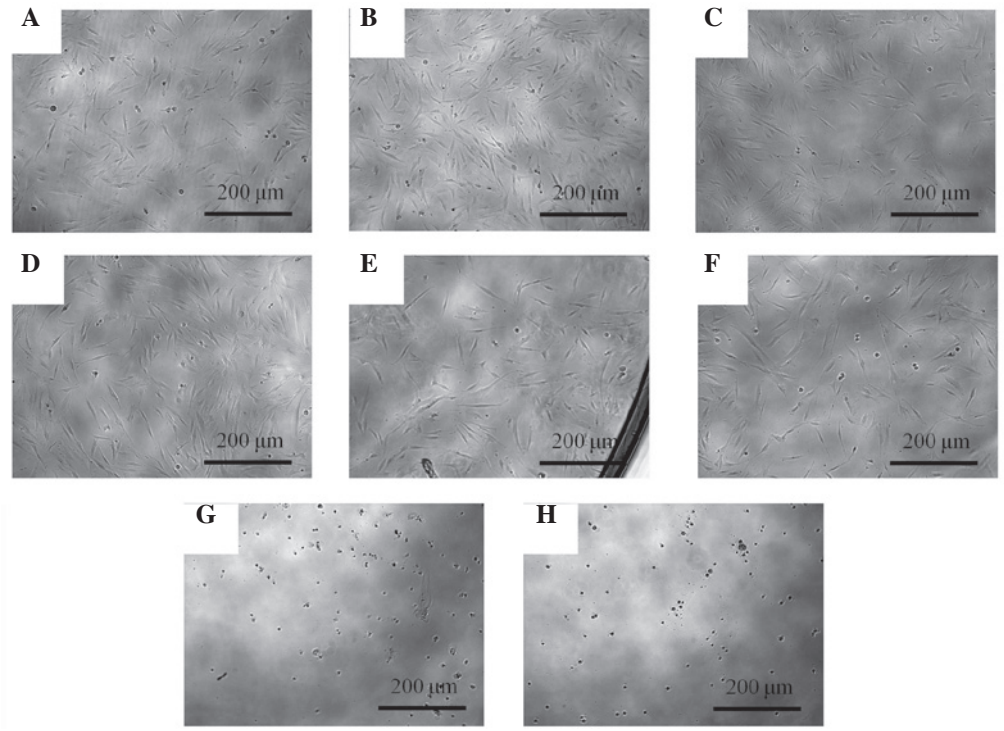

Figure 1. Evaluation of cell morphology on day 1. (A) Control and (B) 0.001 μg/ml, (C) 0.01 μg/ml, (D) 0.1 μg/ml, (E) 1 μg/ml, (F) 10 μg/ml, (G) 100 μg/ml and (H) 1,000 μg/ml Cimicifugae Rhizoma treatment groups.

was concentrated to 300 ml using a rotary evaporator under reduced pressure (Eyela NE-1001; Tokya Rikakikai Co., Ltd, Tokyo, Japan). The concentrates were then freeze-dried using a lyophilizer (Labconco, Kansas, MO, USA) to obtain 92.8 g solid residue, resulting in a yield of 18.6% (w/w).

Isolation and culture of stem cells derived from gingiva. Healthy gingival tissues were obtained from healthy patients undergoing crown-lengthening procedures. This study was reviewed and approved by the Institutional Review Board of Seoul St. Mary's Hospital, College of Medicine, The Catholic University of Korea (Seoul, Korea; KC11SISI0348), and informed consent was obtained from all patients.

The tissues were immediately placed in sterile phosphate-buffered saline (PBS; Welgene, Inc., Daegu, Korea) with 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA) at 4˚C. The gingival tissue was de-epithelialized, minced, digested with collagenase IV (Sigma-Aldrich) and incubated at 37˚C in a humidified incubator with 5% CO_2 and 95% O_2. The non-adherent cells were washed with PBS after 24 h, supplied with essential minimal medium α (α-MEM; Gibco Life Technologies, Grand Island, NY, USA) containing 15% fetal bovine serum (Gibco), 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma-Aldrich), 200 mM L-glutamine (Sigma-Aldrich) and 10 mM ascorbic acid 2-phosphate (Sigma-Aldrich), and fed every 2-3 days. These cells showed the characteristics of stem cells, including colony-forming abilities, plastic adherence and multi-lineage differentiation (osteogenic, adipogenic, chondrogenic) potency. Approximately $3x10^5$ cells were incubated with specific PE-, APC-, BV421-, PerCP-cyTM5.5- or fluorescein isothiocyanate-conjugated mouse monoclonal antibodies for human CD44, CD73, CD90, CD105, CD14, CD45, CD34 and CD19 (BD Biosciences, San Jose, CA, USA). The cells expressed CD44, CD73, CD90 and CD105, but did not express CD14, CD45, CD34 and CD19 when examined by flow cytometry (FACSCanto II; BD Biosciences).

Evaluation of stem cell morphology. The stem cells were plated at a density of $2.0x10^3$ cells/well in 96-well plates. The cells were incubated in α-MEM (Gibco Life Technologies, Grand Island, NY, USA) that was composed of 15% fetal bovine serum (Gibco Life Technologies), 100 U/ml penicillin, 100 μg/ml streptomycin, 200 mM L-glutamine (Sigma-Aldrich) and 10 mM ascorbic acid 2-phosphate (Sigma-Aldrich) in the presence of the Cimicifugae Rhizoma extract at final concentrations that ranged from 0.001 to 1,000 μg/ml. The concentrations used were 0 (untreated control), 0.001, 0.01, 0.1, 1, 10, 100 and 1,000 μg/ml, respectively. The morphology of the cells was viewed under an inverted microscope (Leica DM IRM; Leica Microsystems, Wetzlar, Germany) on days 1, 3, 5 and 7. The images were saved as JPEG files.

Determination of cell proliferation. The analysis of cell proliferation was performed on days 1, 3, 5 and 7. Viable cells were identified using a Cell Counting kit-8 (CCK-8; Dojindo Molecular Technologies, Inc.) assay. The spectrophotometric absorbance was measured with a Synergy MX microplate reader (BioTek Instruments, Inc.; Winooski, VT, USA), and the analysis was performed in triplicate.

Statistical analysis. The results are represented as the means ± standard deviation. A test of normality was performed, and a one-way analysis of variance (ANOVA) with post hoc

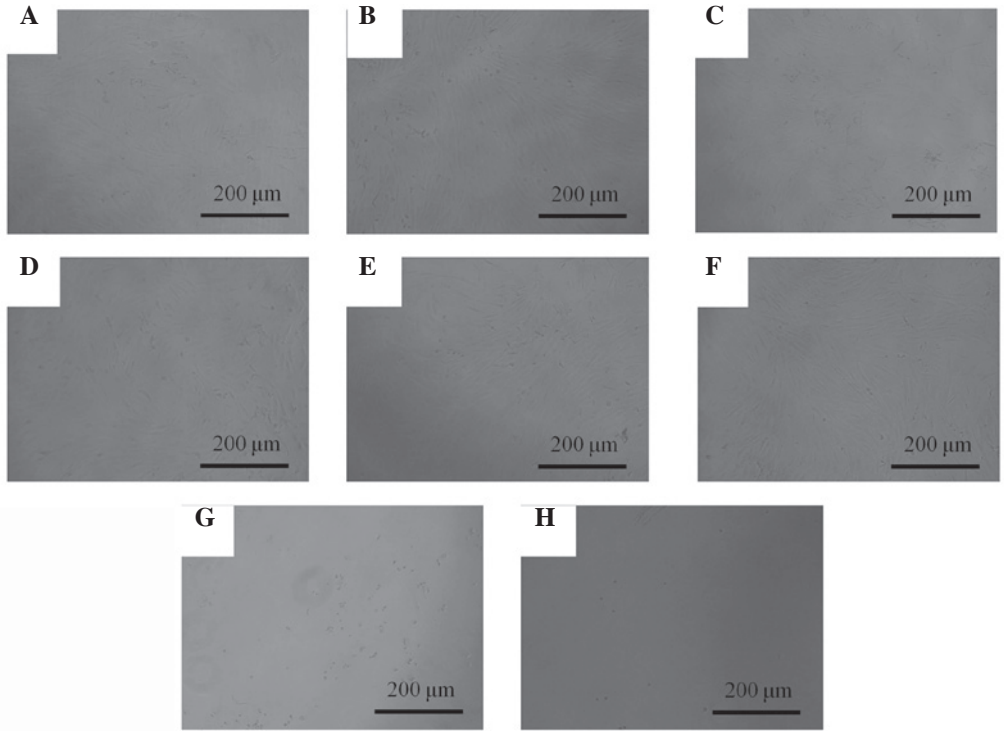

Figure 2. Cell morphology on day 3. (A) Control and (B) 0.001 μg/ml, (C) 0.01 μg/ml, (D) 0.1 μg/ml, (E) 1 μg/ml, (F) 10 μg/ml, (G) 100 μg/ml and (H) 1,000 μg/ml Cimicifugae Rhizoma treatment groups.

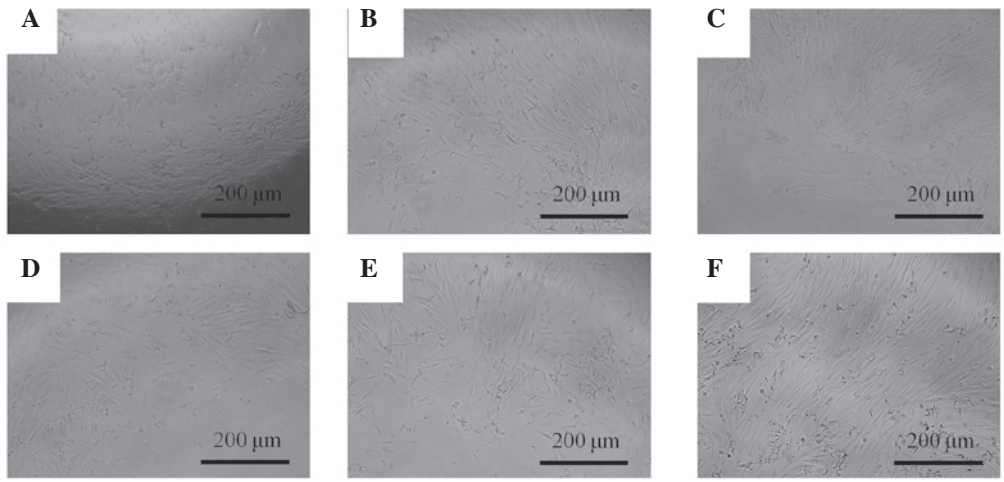

Figure 3. Cell morphology on day 5. (A) Control and (B) 0.001 μg/ml, (C) 0.01 μg/ml, (D) 0.1 μg/ml, (E) 1 μg/ml and (F) 10 μg/ml Cimicifugae Rhizoma treatment groups.

test was performed to determine the differences between the groups using commercially available software (SPSS version 12 for Windows; SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Evaluation of cell morphology. The morphology of the stem cells at day 1 is shown in Fig. 1. Under an optical microscope, the control group showed spindle-shaped, fibroblast-like morphology. The shapes of the cells treated with 0.001-10 μg/ml Cimicifugae Rhizoma were similar to the shapes of the control group. Significant alterations were noted in the 100 and 1,000 μg/ml groups when compared with the control group. The shapes of the cells in the 100 and 1,000 μg/ml groups were rounder, and fewer cells were present.

The morphology of the cells on day 3 is shown in Fig. 2. The shapes of the cells in treated with 0.001-10 μg/ml were similar to the shapes of the control group. Marked alterations in cytoskeletal organization were observed in the 100 and 1,000 μg/ml groups. The shapes of the cells in the 100 and 1000 μg/ml groups were rounder, and fewer cells were present, when compared with the control group.

The morphology of the cells on days 5 and 7 is shown in Figs. 3 and 4, respectively. The shapes of the cells in the

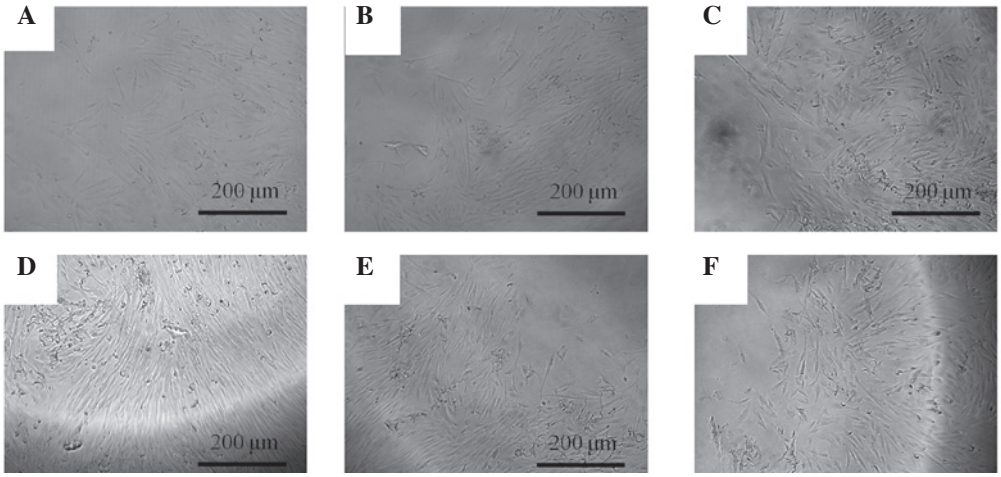

Figure 4. Cell morphology on day 7. (A) Control and (B) 0.001 μg/ml, (C) 0.01 μg/ml group. (D) 0.1 μg/ml, (E) 1 μg/ml group and (F) 10 μg/ml Cimicifugae Rhizoma treatment groups.

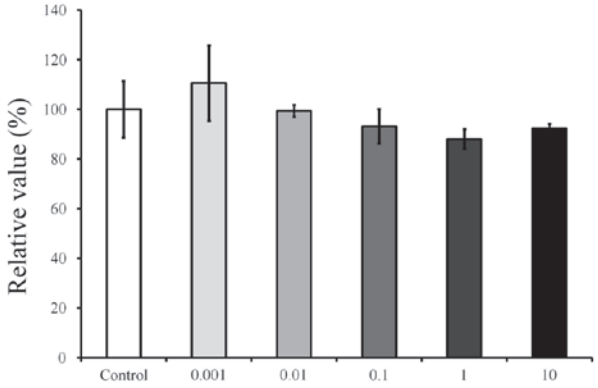

Figure 5. Cellular viability on day 1.

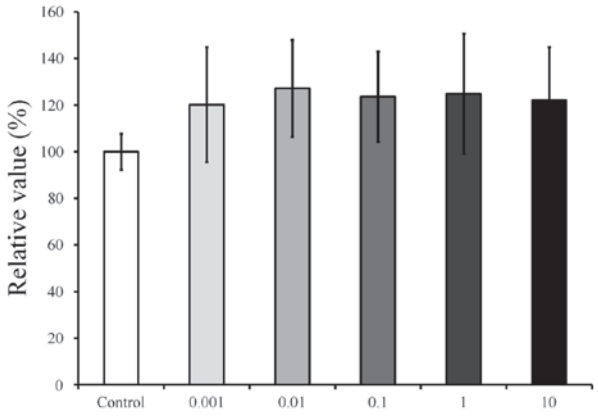

Figure 6. Cellular viability on day 3.

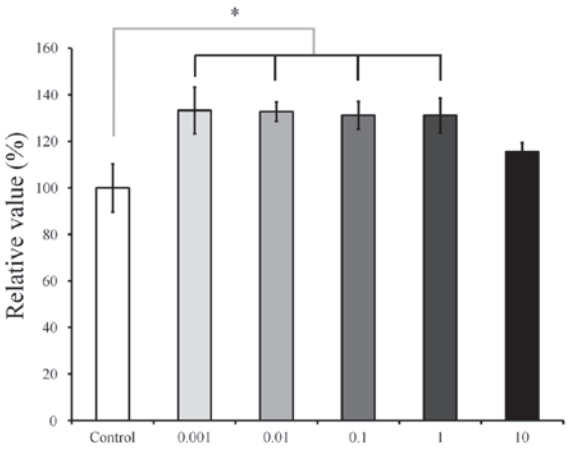

Figure 7. Cellular viability on day 5. *P<0.05, statistically significant differences between the control and treatment groups.

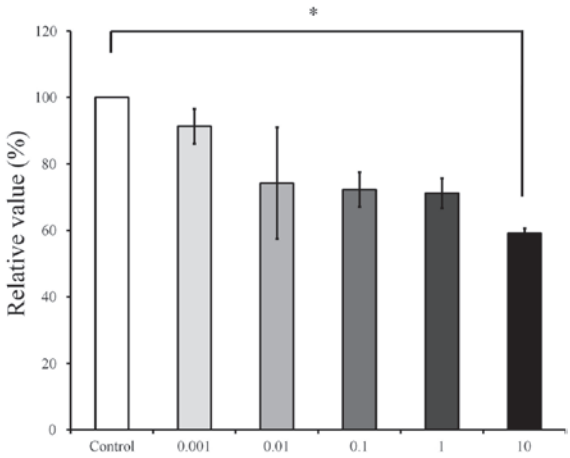

Figure 8. Cellular viability on day 7. *P<0.05, statistically significant differences between the control and treatment groups.

0.001-10 μg/ml groups were similar to the shapes of the untreated control group.

Cell proliferation. The results of the cell proliferation assay on days 1, 3, 5 and 7 are shown in Fig. 5, 6, 7 and 8, respectively. The cultures that were grown in the presence of Cimicifugae Rhizoma on day 1 showed an increase in the CCK-8 result at 0.001 μg/ml (Fig. 5). The relative value of the CCK-8 assay result for 0.001 μg/ml Cimicifugae Rhizoma was 110.6±15.2%, when the CCK-8 result of the untreated control group on day 1 was considered to be 100%; however, there were no significant differences (P>0.05). On day 3 (Fig. 6), the relative value of

the CCK-8 result for 0.001 μg/ml Cimicifugae Rhizoma was 120.2±24.6%, when the CCK-8 result of the untreated control group on day 3 was considered to be 100%. On day 5 (Fig. 7), the cultures grown in the presence of Cimicifugae Rhizoma at concentrations of 0.001, 0.01, 0.1 and 1 μg/ml resulted in increased CCK-8 values ($P<0.05$). The relative values of the CCK-8 results for 0.001, 0.01, 0.1, 1 and 10 μg/ml Cimicifugae Rhizoma were 133.3±10.0, 132.7±4.2, 131.2±5.8, 131.2±7.3 and 115.5±4.0%, respectively, when the CCK-8 result of the untreated control group on day 5 was considered to be 100% (100.0±7.7%). On day 7 (Fig. 8), The relative value of the CCK-8 result for 10 μg/ml Cimicifugae Rhizoma was 59.2±1.5%, when the CCK-8 result of the untreated control group on day 7 was considered to be 100% ($P<0.05$).

Discussion

In this study, the effects of Cimicifugae Rhizoma on the morphology and proliferation of human mesenchymal stem cells derived from periodontal tissue were investigated. The results clearly demonstrated that the stem cells were sensitive to Cimicifugae Rhizoma at high concentrations and that a significant reduction in cellular viability occurred at 100 and 1,000 μg/ml concentrations.

The effects of Cimicifugae Rhizoma have previously been tested in *in vitro* and *in vivo* experiments (10-12). A Cimicifugae Rhizoma extract has shown cytotoxicity toward human cancer cell lines, including promyelocytic, lung carcinoma and human colon adenocarcinoma cell lines (12). The cytotoxicity of Cimicifugae Rhizoma has been tested and the half maximal inhibitory concentration (IC_{50}) values on hepatocellular carcinoma and drug-resistant hepatocellular carcinoma cell lines and primary cultured normal mouse hepatocytes were found to be 21, 43 and 80 μg/ml, respectively (10). In a rat model, Cimicifugae Rhizoma extract at a dosage of 50 mg/kg showed a slight toxicity in the liver and kidney via disturbance of the metabolisms of energy and amino acids, which provides a reasonable explanation for the clinical hepatotoxicity (11).

Cimicifugae Rhizoma is a traditional herbal medicine used to treat various diseases. The anticancer properties of plants of the genus *Cimicifuga* have received considerable attention in recent years (12). Cimicifugae Rhizoma can be used for the treatment of cardiovascular disorders such as atherosclerosis (13). Ovariectomized rats treated with extracts of Cimicifugae Rhizoma exhibited a significant increase in bone mineral density compared with that in untreated rats (2), and it was suggested that these anti-bone resorption effects of Cimicifugae Rhizoma may be applied therapeutically against osteoporosis (14,15). Aqueous extracts of Cimicifugae Rhizoma have shown central nervous system effects by binding to the 5-HT1A receptor (4). Cimicifugae Rhizoma has also demonstrated inhibitory effects on histamine, bradykinin and COX-2-mediated inflammatory actions (5). The analgesic and sedative effects of Cimicifugae Rhizoma have been noted using animal model experiments (16). As an effective antioxidant, Cimicifugae Rhizoma can protect deoxyribonucleic acid and lipids against oxidative damage, and its antioxidant ability may be responsible for its various pharmacological effects (17). Cimicifugae Rhizoma has also been reported to protect the intestines and hematopoietic organs against radiation damage (18). A herbal drug containing multiple medicinal plants, including Cimicifugae Rhizoma, has demonstrated the ability to decrease bacterial counts in urine culture (19), and Cimicifugae Rhizoma has also shown an antiviral effect (20).

There is great interest in stem cells due to their promising potential for the treatment of diseases and the regeneration of tissue (21). Stem cells may be obtained from various tissues, including bone marrow and adipose tissue (22,23). Moreover, stem cells may be obtained intraorally, including from dental pulp and periodontal ligaments (24,25). However, tissue obtained intraorally may not be easily accessible, and only a limited amount of the tissue can be obtained in a limited number of procedures. By contrast, the gingiva is an readily accessible tissue source (26). Thus, stem cells derived from gingiva may be useful for the research and treatment of disease.

Within the limits of the present study, Cimicifugae Rhizoma influenced the viability of stem cells derived from gingiva, and its direct application onto oral tissues may produce adverse effects at high doses. The concentration and application time of Cimicifugae Rhizoma should be meticulously controlled to obtain optimal results.

Acknowledgements

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