

Effects of *Asiasari radix* on the morphology and viability of mesenchymal stem cells derived from the gingiva

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Received November 24, 2013; Accepted June 17, 2014

DOI: 10.3892/mmr.2014.2607

Abstract. Medicinal herbs used in traditional Oriental medicine, which have been in use clinically for thousands of years, are attractive sources of novel therapeutics or preventatives. *Asiasari radix* (*A. radix*) has been suggested for use in the treatment of dental diseases, including toothache and aphthous stomatitis. The aim of this study was to evaluate the effects of *A. radix* extracts on the morphology and viability of human stem cells derived from the gingiva. An *Asiasarum heterotropoides* extract was centrifuged and freeze-dried in a lyophilizer. Stem cells derived from the gingiva were grown in the presence of *A. radix* at concentrations ranging between 0.1 $\mu\text{g/ml}$ and 1 mg/ml (0, 0.1, 1, 10, 100 and 1,000 $\mu\text{g/ml}$). Cell morphology was evaluated with an optical microscope and the viability of the cells was quantitatively analyzed with a cell counting kit-8 (CCK-8) assay for up to seven days. The untreated control group exhibited normal fibroblast morphology. The shapes of the cells following 0.1, 1, 10 and 100 $\mu\text{g/ml}$ *A. radix* treatments were similar to those of the control group. However, a significant change was noted in the 1,000 $\mu\text{g/ml}$ group on day 1, when compared with the untreated group. Furthermore, on day 7, the shapes of the cells following 100 and 1,000 $\mu\text{g/ml}$ *A. radix* treatments were rounder and fewer cells were present, when compared with those of the control group. The cultures that grew in the presence of *A. radix* did not exhibit any changes in the CCK-8 assay on day 2; however, significant reductions in cell viability were noticed following 100 and 1,000 $\mu\text{g/ml}$ *A. radix* treatment on days 5 and 7. Within the limits of this study, *A. radix* influenced the viability of the stem cells derived from the gingiva. Thus, the direct application of *A. radix* to oral tissues may produce adverse effects at high doses.

Therefore, the concentration and application time of *A. radix* requires meticulous control to obtain optimal results. These effects require consideration, if the use of *A. radix* is planned for the treatment of dental diseases.

Introduction

Medicinal herbs, which have been used for thousands of years in traditional Oriental medicine, are attractive sources of novel therapeutics or preventives (1,2). The herbs have been pre-validated for effectiveness and are expected to have fewer safety issues than chemically synthesized drugs (3).

Asiasari radix (*A. radix*) has been used as a flavoring substance in a wide variety of dietary products, and as an ingredient in drinks, cosmetics, soaps, shampoos, fragrances and herbal products, without reported adverse effects (4). *A. radix* is termed *seshin* in Korean, *xì xīn* in Chinese and *saishin* in Japanese, and is used for treating pain, allergies and inflammatory disorders in traditional Oriental medicine (3,5,6). *A. radix* is primarily derived from either *Asiasarum heterotropoides* or *Asiasarum sieboldii* (6). Studies has been performed regarding the anti-inflammatory and anti-allergy effects of *A. radix* (7,8).

A. radix has been suggested for use in the treatment of oral diseases, including toothache and aphthous stomatitis (9,10), as this therapy been suggested to exert antibacterial and anti-inflammatory effects (3,6). However, only limited information is currently available regarding the effects of *A. radix* on dental tissue (11) and no information is available on the effects on mesenchymal stem cells derived from the gingiva.

The aim of the present study was to evaluate the effects of *Asiasarum heterotropoides* extract on the morphology and viability of the human stem cells derived from the gingiva.

Materials and methods

Preparation. The dry roots of *Asiasarum heterotropoides* (400 g) were immersed in distilled water and boiled under reflux for 150 min. The resulting extract was centrifuged at 5,000 \times g for 10 min, and the supernatant 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 in a lyophilizer

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Key words: herbal medicine, plant roots, stem cells, cell survival

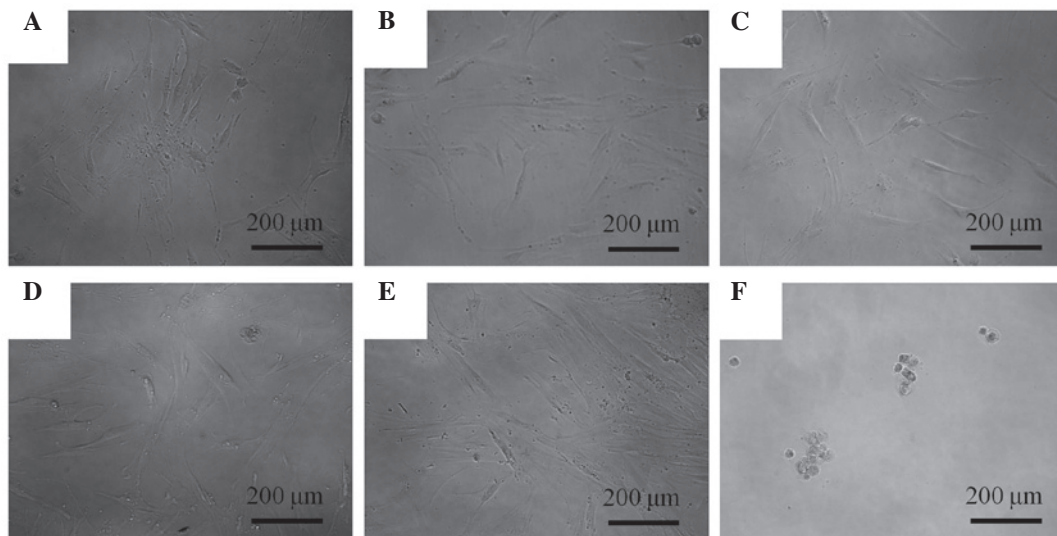


Figure 1. Evaluation of gingival cell morphology on day 1 following: (A) Control, (B) 0.1 $\mu\text{g/ml}$, (C) 1 $\mu\text{g/ml}$, (D) 10 $\mu\text{g/ml}$, (E) 100 $\mu\text{g/ml}$ and (F) 1,000 $\mu\text{g/ml}$ A. radix treatment.

(Labconco, Kansas, MO, USA) to obtain 65 g solid residue (yield 16%, w/w).

Isolation and culture of stem cells derived from the gingiva. Healthy gingival tissue samples were collected from healthy patients undergoing clinical crown lengthening procedures. The design of 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, Republic of Korea (KC11SISI0348) and informed consent was obtained from the patients.

The gingival tissue was de-epithelialized, minced and digested with collagenase IV (Sigma-Aldrich, St. Louis, MO, USA). The cells were incubated at 37°C in a humidified incubator with 5% CO₂ and 95% O₂. After 24 h, any non-adherent cells were washed with phosphate-buffered saline (Welgene, Daegu, Korea) and fresh medium was added. The media was changed every 2-3 days.

Determination of cell viability. The cells were plated at a density of 2.0×10^3 cells/well in 96-well plates. The cells were incubated in minimum essential medium- α containing 15% fetal bovine serum (Gibco-BRL, Carlsbad, CA, USA), 100 U/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin (Sigma-Aldrich), 200 mm L-Glutamine (Sigma-Aldrich) and 10 mm ascorbic acid 2-phosphate (Sigma-Aldrich) in the presence of A. radix at concentrations of: 0 (untreated control), 0, 0.1, 1, 10, 100 and 1,000 $\mu\text{g/ml}$, respectively. 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium, monosodium salt (WST-8; Cell Counting kit-8 CCK-8; Dojindo, Tokyo, Japan) was then added and the cells were incubated for 1 h at 37°C. The analysis was performed on days 2, 3, 5 and 7. Viable cells were identified using the CCK-8 assay, which relies on the ability of mitochondrial dehydrogenases to oxidize WST-8 to a formazan product. The spectrophotometric absorbance at 450 nm was measured using a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). The experiments were performed in triplicate.

Evaluation of cellular morphology. 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 JPEGs.

Statistical analysis. The data are presented as the mean \pm standard deviation. A one-way analysis of variance with post hoc test was performed to determine the differences among the groups using a commercially available program (SPSS 12 for Windows; SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Cell morphology. The control group exhibited normal fibroblast morphology on day 1 (Fig. 1A). The shapes of the cells following 0.1, 1, 10 and 100 $\mu\text{g/ml}$ A. radix treatment were similar to those of the control group (Fig. 1B-E). A significant alteration was noted in the 1,000 $\mu\text{g/ml}$ group when compared with the untreated group. The shapes of the cells in 1,000 $\mu\text{g/ml}$ group were rounder and fewer cells were present (Fig. 1F).

The morphology of the cells on day 3 is shown in Fig. 2. The shapes of the cells subsequent to 0.1, 1, 10 and 100 $\mu\text{g/ml}$ A. radix treatment remained similar to those of the control group. The morphologies of the cells on days 5 and 7 are shown in Figs. 3 and 4, respectively. Marked alterations in cytoskeletal organization were noticed in the 100 and 1,000 $\mu\text{g/ml}$ A. radix groups. The shapes of these cells were rounder, and fewer cells were present, when compared with those of the control group.

Cellular viability. The CCK-8 results on days 2, 3, 5 and 7 are shown in Figs. 5, 6, 7 and 8, respectively. The cultures growing in the presence of A. radix did not exhibit any changes in the CCK-8 assays on day 2 and no significant differences were identified among the six groups (Fig. 5). However, on day 3, a significant reduction in cell viability was observed in the groups with higher concentrations A. radix treatment (1, 10, 100 and 1,000 $\mu\text{g/ml}$), when compared with the cells

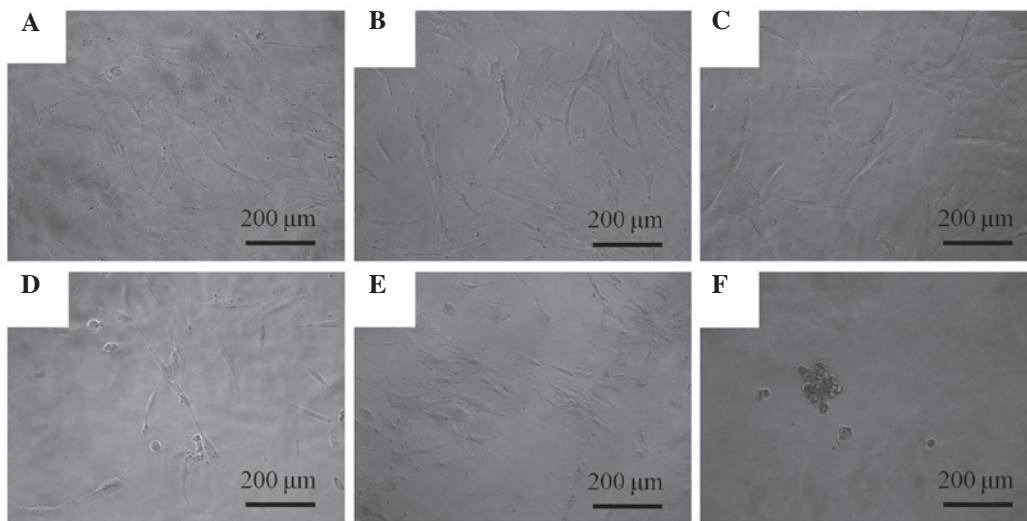


Figure 2. Gingival cell morphology on day 3 following: (A) Control, (B) 0.1 μg/ml, (C) 1 μg/ml, (D) 10 μg/ml, (E) 100 μg/ml and (F) 1,000 μg/ml *A. radix* treatment.

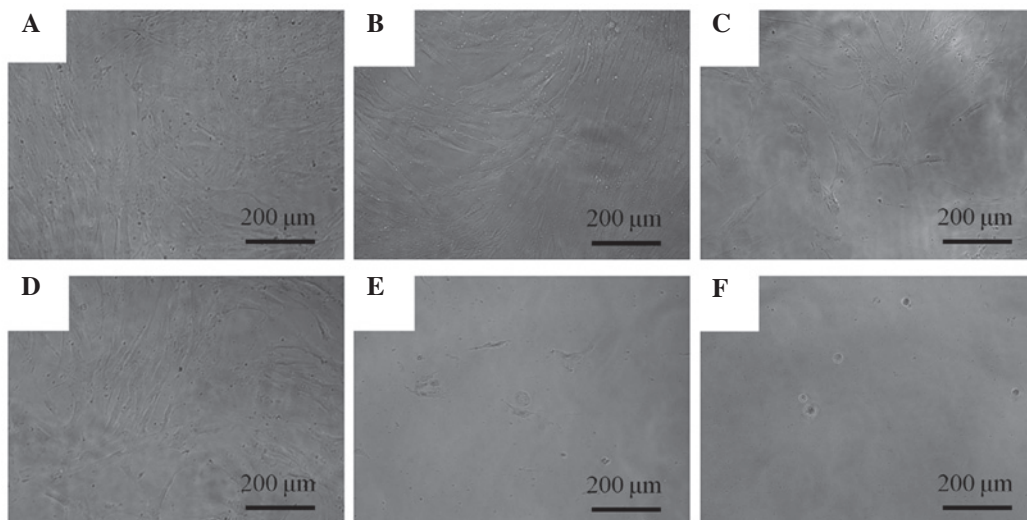


Figure 3. Evaluation of gingival cell shape on day 5 following: (A) Control, (B) 0.1 μg/ml, (C) 1 μg/ml, (D) 10 μg/ml, (E) 100 μg/ml and (F) 1,000 μg/ml *A. radix* treatment.

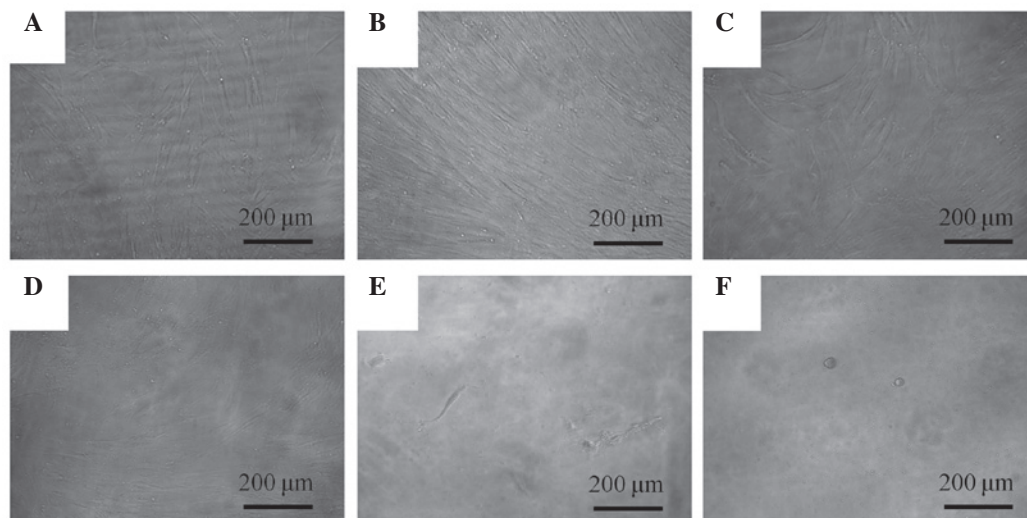


Figure 4. Evaluation of gingival cell morphology on day 7 following (A) Control, (B) 0.1 μg/ml, (C) 1 μg/ml, (D) 10 μg/ml, (E) 100 μg/ml and (F) 1,000 μg/ml *A. radix* treatment.

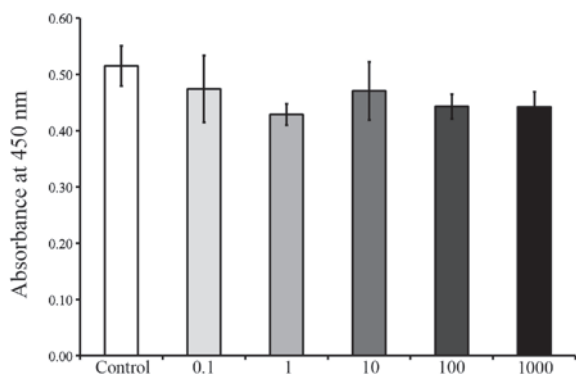


Figure 5. Gingival cell viability on day 2 following 0.1, 1, 10 and 100 µg/ml A. radix treatment.

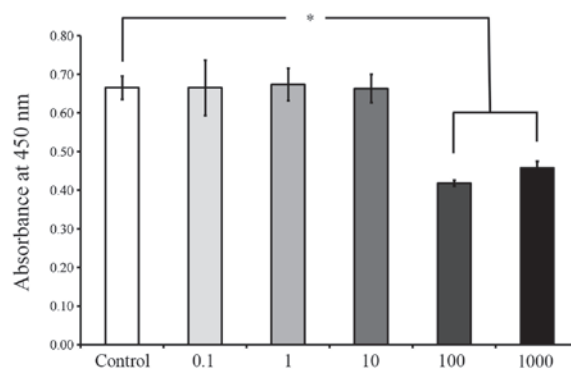


Figure 8. Gingival cell viability on day 7 following 0.1, 1, 10 and 100 µg/ml A. radix treatment. *P<0.05, as compared with the control group.

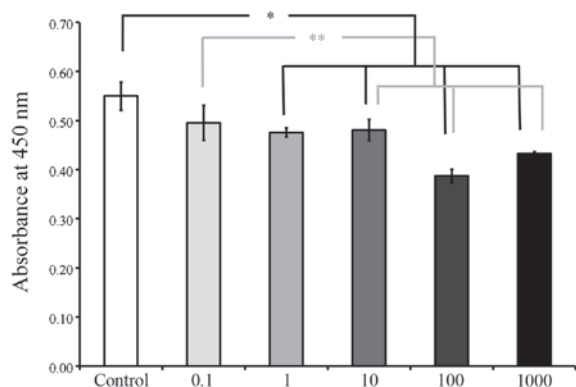


Figure 6. Gingival cell viability on day 3 following 0.1, 1, 10 and 100 µg/ml A. radix treatment. *P<0.05, as compared with the control group; **P<0.05, as compared with the 0.1 µg/ml group.

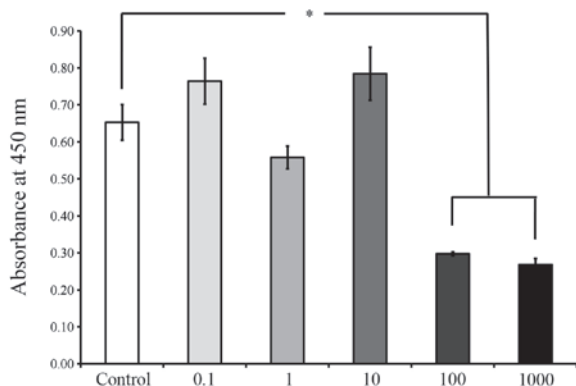


Figure 7. Gingival cell viability on day 5 following 0.1, 1, 10 and 100 µg/ml A. radix treatment. *P<0.05, as compared with the control group.

in the control group (P<0.05; Fig. 6). The viability results on days 5 and 7, as shown in Figs. 7 and 8, respectively, revealed significant reductions in cell viability following 100 and 1,000 µg/ml A. radix treatment (P<0.05; Figs. 7 and 8), when compared with the control cells.

Discussion

In this report, the effects of A. radix on the morphology and cell viability of stem cells derived from the gingiva under

predetermined concentrations were examined. High concentrations of A. radix exerted adverse effects on the gingival stem cells, and a significant reduction in cellular viability was observed at 100 and 1,000 µg/ml A. radix concentrations.

The effects of A. radix have been previously analyzed in *in vitro* and *in vivo* experiments (4,12,13). One study revealed no significant effect on the growth of HeLa cells following A. radix treatment for 72 h at a range of concentrations between 0.0001 and 1,000 µg/ml (12). The safety of an A. radix methanol extract was investigated in an oral sub-acute 28-day toxicity study in Sprague-Dawley rats at doses of 50, 250 and 500 mg/kg/day, and the authors concluded that the methanolic extract of A. radix appeared to be safe and nontoxic within the experimental conditions (4). The acute oral toxicity of A. radix methanol extract was evaluated in ICR mice; the results revealed no cases of mortality, signs of toxicity or abnormalities in the gross findings (13). The authors concluded that the methanol extract of A. radix was toxicologically safe for oral administration. However, the results of the present study demonstrated that gingival cells treated with 100 and 1,000 µg/ml A. radix exhibited cell damage with significant reductions in cell viability on days 3, 5 and 7. The conflicting results regarding the different responses to A. radix may, in part, be attributed to the type of cells, culturing period or culturing conditions (14).

In the present study, a CCK-8 assay utilizing water-soluble tetrazolium salt-8 was used to evaluate the viability of the gingival cells. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is reported to be a more sensitive assay than trypan blue (15), which is determined by the principle that viable cells have intact cell membranes that exclude the trypan blue dye (16). Dead cells absorb trypan blue and appear blue as a consequence, as the cell membranes are unable to control the passage of macromolecules. The MTT assay determines cellular viability by determining mitochondrial dehydrogenase activity; however, further treatment is required to solubilize the formazan crystals and the MTT agent may be toxic to the cells (17). CCK-8 assay has been reported to be more sensitive than the MTT assay and less toxic to cells (17).

Increasing interest has been generated in the potential of stem cells, which are promising candidates for the regeneration of tissues and the treatment of diseases (18). Mesenchymal stem cells (MSCs) may be isolated from various tissues, including bone marrow (19), adipose tissue (20) and

muscle (21). Recently, MSCs have been identified in various oral tissues, including dental pulp (22), the lamina propria of the oral mucosa (23) and the periodontal ligaments (24). These tissues may not be easily accessible or obtainable (25); however, the gingiva is easily accessible in dental clinics and, as this tissue is discarded in routine periodontal surgery, may be utilized for the isolation of human MSCs. Thus, stem cells derived from the gingiva may be useful in the investigation and treatment of disease.

A. radix, a traditional herbal medicine commonly used to treat various diseases, has been reported to be safe and nontoxic in previous studies (4,13). A. radix has been shown to treat dental diseases (9,10,26); however, limited information is available regarding the optimal dosage. The present study provided clarification with regard to these issues. A. radix was shown to influence the viability of stem cells derived from the gingiva, with reduced viability at higher concentrations. Therefore, the direct application of A. radix to oral tissues may produce adverse effects at high doses. Thus, the concentration and application time of A. radix requires meticulous control to obtain optimal results.

Acknowledgements

The present study was supported by the Basic Science Research Program, through the National Research Foundation of Korea funded by the Ministry of Science, ICT & Future Planning (NRF-2014R1A1A1003106).

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