Effects of *Bambusa tulda* on the proliferation of human stem cells

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Abstract. To date, the effects of *Bambusa tulda* on stem cells have not been thoroughly assessed. The present study aimed to evaluate the effects of *Bambusa tulda* extract on the morphology and proliferative potential of human mesenchymal stem cells derived from the gingiva. The stem cells were cultured in a growth medium in the presence of *Bambusa tulda* methanolic extract (BBT) at concentrations ranging from 0.001 to 1%. Evaluation of cell morphology and cellular proliferation as well as immunofluorescent assays for collagen I were performed on days 1, 3, 5 and 7. Stem cells in the control group displayed a fibroblast-like morphology, and BBT treatment did not produce any noticeable morphological changes. However, application of 1% BBT produced a significant increase in cell proliferation. BBT, particularly at the concentration of 1%, also caused a noticeable increase of collagen I expression at day 1 and day 3. Based on these findings, it was concluded that BBT exerted beneficial effects on the proliferation of mesenchymal stem cells and enhanced collagen I expression at early time points.

Introduction

Bambusa tulda Roxburgh, also known as Indian timber bamboo, is an evergreen gregarious bamboo with grey or greyish-green culms that is native to the Indian subcontinent, Indochina, Tibet and Yunnan, and naturalized in Puerto Rico and parts of South America (1). *Bambusa tulda* has been used for various purposes and has been widely considered as one of the most useful bamboo species (2). *Bambusa tulda* is widely used in paper pulp industry in Asia (3). *Bambusa tulda* produces nutritive shoots and there is high demand for edible bamboo shoots in many Asian ethnic groups (4).

The leaves of *Bambusa tulda* are 3-4 cm broad and 20-35 cm in length with an oblong-lanceolate shape (5). Medicinal uses of the leaves of *Bambusa tulda* have not yet been widely studied, and the effects of *Bambusa tulda* leaf extract on stem cells remain to be thoroughly assessed. The present study aimed to evaluate the effects of *Bambusa tulda* methanolic extract (BBT) on the morphology and proliferative potential of human mesenchymal stem cells.

Materials and methods

Preparation of plant materials. The *Bambusa tulda* Roxburgh was collected in the village of Amki, Sonaimuri Upazilla, Noakhali District, Bangladesh and a voucher specimen (no. PB022073) was deposited in the herbarium of the Korea Research Institute of Bioscience and Biotechnology. After drying and grinding the leaves of *Bambusa tulda*, the powder (63 g) was added to 500 ml methanol. The extraction was performed using the method of repercolation at room temperature. The extract was filtered and concentrated by a rotavapor under reduced pressure to obtain 2.75 g BBT.

Stem cells isolated from human gingiva. A healthy, 63-year-old, female patient visiting the Department of Periodontics of Seoul St. Mary's Hospital (College of Medicine, the Catholic University of Korea, Seoul, Korea) provided the gingivae for the study. The Institutional Review Board at Seoul St. Mary's Hospital reviewed and gave approval for the study (no. KC11SISI0348), and written informed consent was obtained from the patient. All the methods used in this study were performed in accordance with the ethical standards of the Institutional Research Committee and with the 1964 Helsinki Declaration and its later amendments. The epithelium of the obtained gingiva was removed and the tissue was cut into 1-2 mm fragments. The gingival tissues were digested with media containing dispase (1 mg/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and collagenase IV (2 mg/ml; Sigma-Aldrich; Merck KGaA) (6). Cells that were not attached to the culture dish were removed. The media was changed every 2-3 days and cells were cultured in an incubator with a humidified atmosphere of 5% CO_2 and 95% air at 37˚C.

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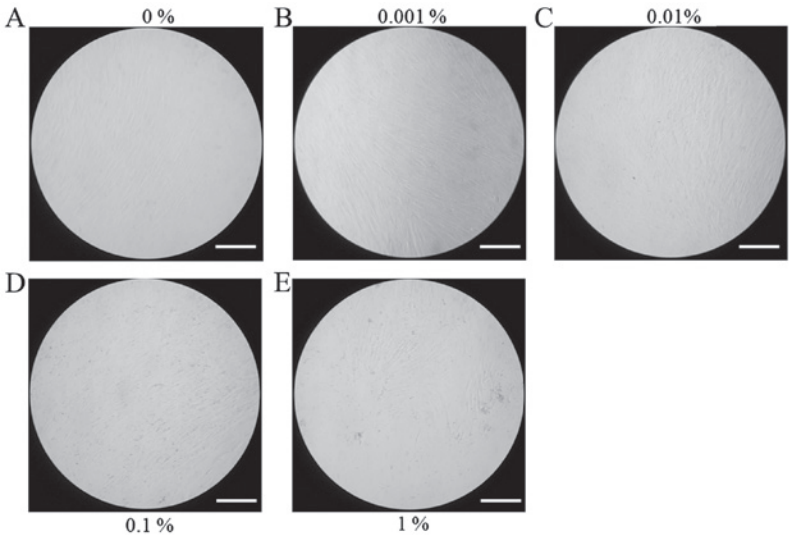

Figure 1. Evaluation of cell morphology on day 1 using inverted microscopy following addition of different concentrations of BBT to the growth media. (A) Control group and (B) 0.001, (C) 0.01, (D) 0.1 and (E) 1% BBT groups (original magnification, x200; scale bar, 200 μm). BBT, *Bambusa tulda* methanolic extract.

Evaluation of cellular morphology. The cells were seeded in 96-well plates at a density of $2.0x10^3$ cells/well and incubated in control medium [α-minimal essential medium (α-MEM); Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA] supplemented with 15% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 200 mM L-glutamine, 10 mM ascorbic acid 2-phosphate, 100 U/ml penicillin and 100 μg/ml streptomycin (all from Sigma-Aldrich; Merck KGaA) in the presence of BBT at final concentrations of 0 (control), 0.001, 0.01, 0.1 and 1%. BBT was dissolved in dimethyl sulfoxide (DMSO) and equal amounts of DMSO (0.1%) were added to each culture sample to offset the influence of this dissolving vehicle. The morphology of the stem cells was evaluated using an inverted microscope (CKX41SF; Olympus Corp., Tokyo, Japan) on days 1, 3, 5 and 7.

Cellular proliferation. Evaluation of the proliferation of the cells grown in medium containing BBT was performed on days 1, 3, 5 and 7 using the Cell Counting Kit-8 (CCK-8; Dojindo, Tokyo, Japan) assay. Tetrazolium monosodium salt was added to the culture, followed by incubation at 37˚C for 2 h. The spectrophotometric absorbance at 450 nm was determined using a microplate reader (BioTek Instruments Inc., Winooski, VT, USA).

Immunofluorescence. Collagen I antibody (cat. no. ab76956; Abcam, Cambridge, UK) was used for the immunofluorescent assay performed on days 1, 3, 5 and 7. Following the specific incubation with BBT, the cells were fixed, permeabilized, blocked and incubated with primary antibodies to collagen I (1:67) overnight at 4˚C. The cultures were incubated for two h at room temperature with fluorescein isothiocyanate-conjugated secondary antibody (cat. no. F2761; 1:100; Thermo Fisher Scientific, Inc.) and then stained with DAPI. A fluorescence microscope (Axiovert 200; Zeiss, Jena, Germany) was used for the analyses.

Statistical analysis. Values are expressed as the mean ± standard deviation of the experiments. A test of normality was performed and one-way analysis of variance with Scheffe's test post hoc test was performed to determine differences between the groups with commercially available software (SPSS 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 stem cells treated with BBT at final concentrations of 0, 0.001, 0.01, 0.1 and 1% on day 1 is presented in Fig. 1. Stem cells in the control group had a fibroblast-like morphology on day 1 (Fig. 1A). No noticeable differences in the morphology of stem cells treated with BBT were observed when compared with that in the untreated control group (Fig. 1B-E). The results on the cell morphology at days 3, 5 and 7 are presented in Figs. 2-4, respectively. Cells appeared to be more densely gathered in the 1% group at day 5 and 7 compared with other groups at the same time points.

Cellular proliferation. The results of the CCK-8 assay revealing the effects of BBT on cellular viability on days 1, 3, 5 and 7 are presented in Fig. 5. After 1 day of culture, the number of viable cells in the 0.001, 0.01, 0.1 and 1% BBT groups was 112.5±17.5, 121.2±14.3, 126.9±10.5 and 157.0±13.1% of that of the control group, respectively ($P<0.05$). On day 3, the relative number of viable cells in the 0.001, 0.01, 0.1 and 1% BBT groups was 103.4±12.5, 116.7±15.1, 121.6±4.2 and 184.4±13.3%, respectively, of that in the control group ($P<0.05$). The relative number of viable cells in the 0.001, 0.01, 0.1 and 1% groups on day 5 was 110.4±10.6, 113.4±14.8, 102.6±5.2 and 136.4±7.7% of the control group, respectively ($P<0.05$). On day 7, the relative number of viable cells in the 0.001, 0.01, 0.1 and 1% groups was 79.8±3.7, 75.0±2.4, 76.8±4.3 and 70.9±1.6%, respectively, compared with that in the control group.

Immunofluorescence. Representative immunofluorescence images for collagen I staining on days 1, 3, 5 and 7 for

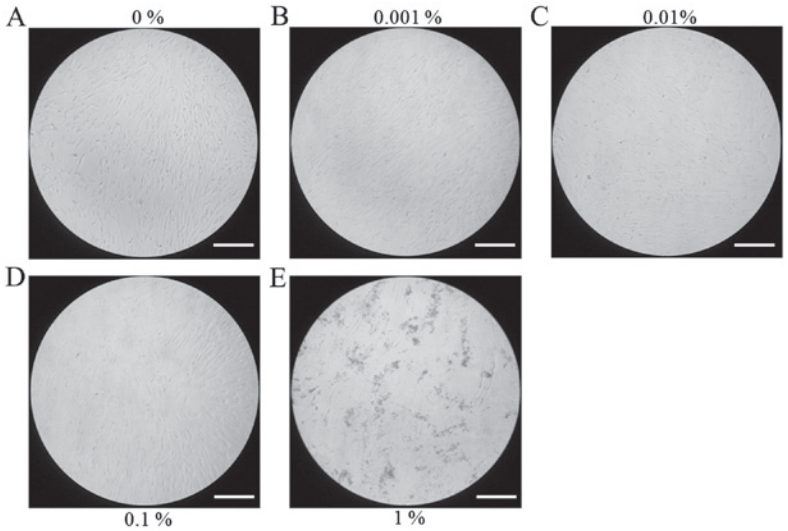

Figure 2. Evaluation of cell morphology on day 3 following addition of different concentrations of BBT to the growth media. (A) Control group and (B) 0.001, (C) 0.01, (D) 0.1 and (E) 1% BBT groups (original magnification, x200; scale bar, 200 μm). BBT, *Bambusa tulda* methanolic extract.

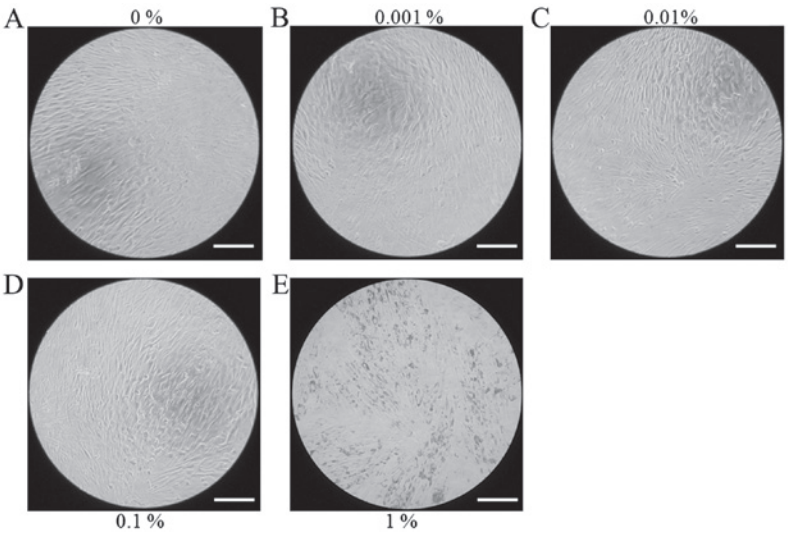

Figure 3. Evaluation of cell morphology on day 5 following addition of different concentrations of BBT to the growth media. (A) Control group and (B) 0.001, (C) 0.01, (D) 0.1 and (E) 1% BBT groups (original magnification, x200; scale bar, 200 μm). BBT, *Bambusa tulda* methanolic extract.

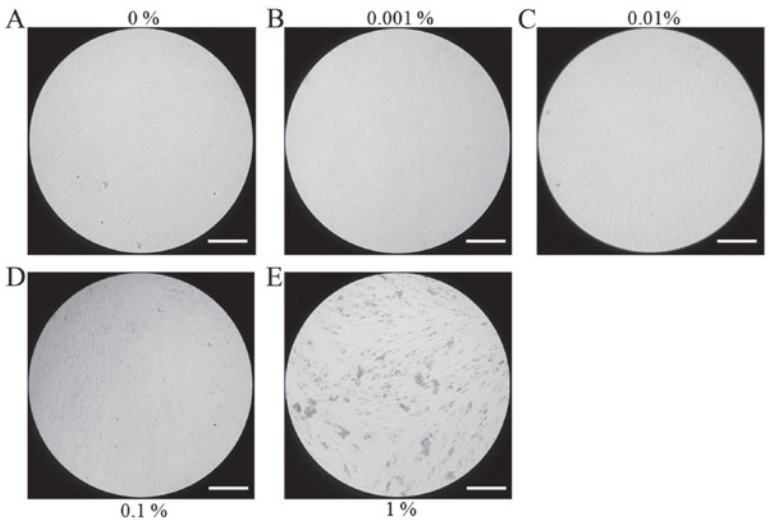

Figure 4. Evaluation of cell morphology on day 7 following addition of different concentrations of BBT to the growth media. (A) Control group and (B) 0.001, (C) 0.01, (D) 0.1 and (E) 1% BBT groups (original magnification, x200; scale bar, 200 μm). BBT, *Bambusa tulda* methanolic extract.

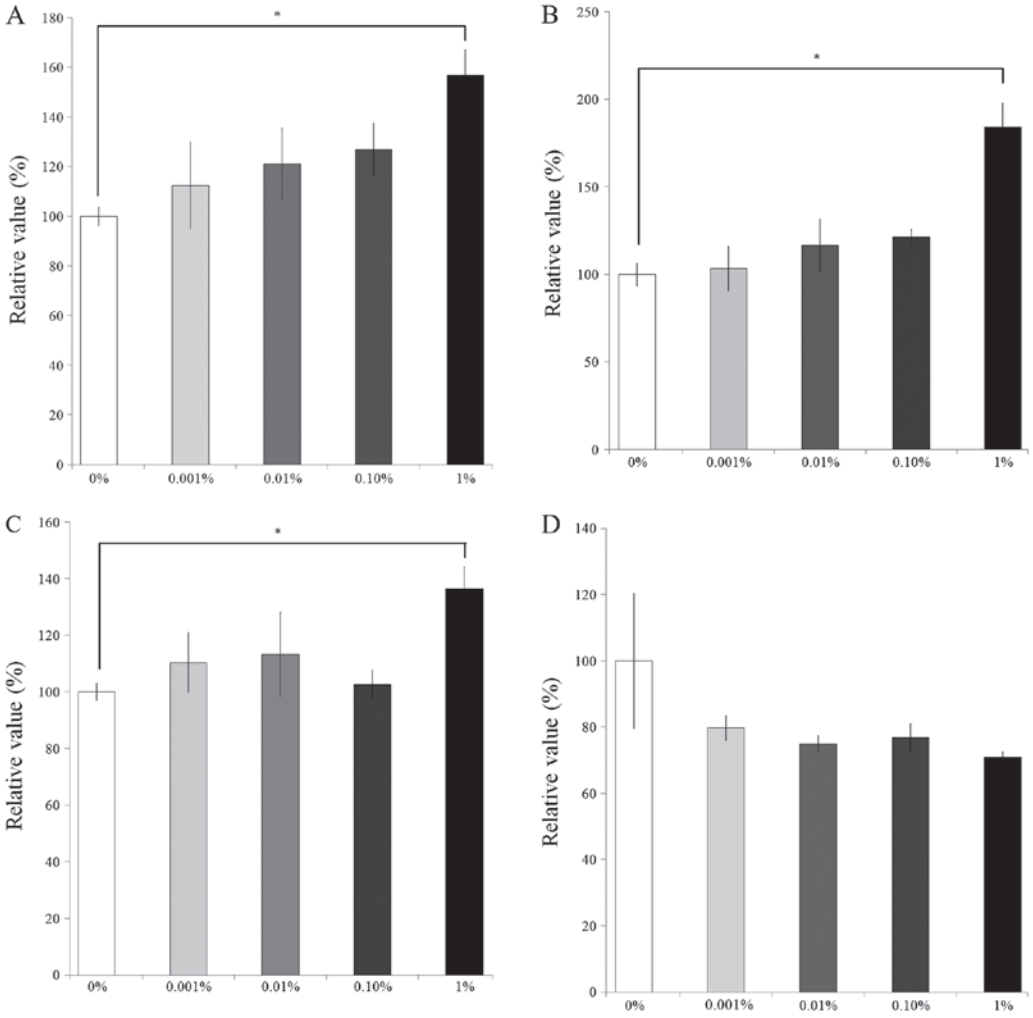

Figure 5. The Cell Counting Kit-8 assay results for stem cells cultured with various concentrations of *Bambusa tulda* methanolic extract for (A) 1, (B) 3, (C) 5 and (D) 7 days. *P<0.05 vs. untreated control.

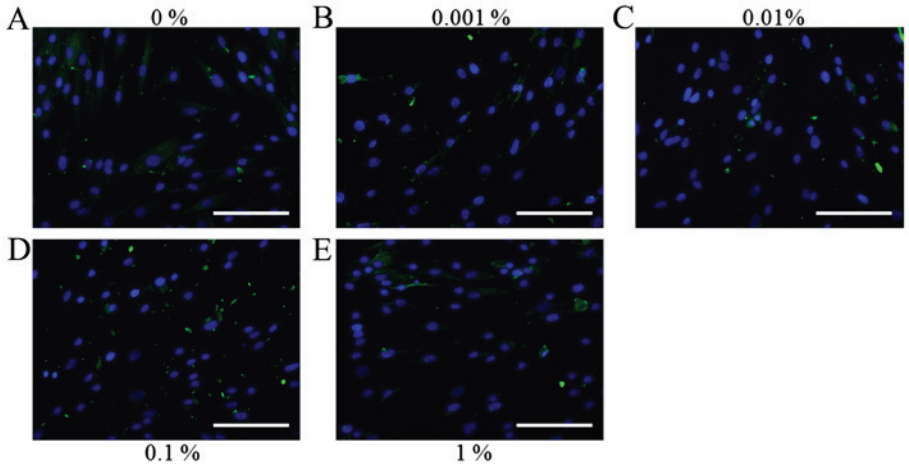

Figure 6. Immunofluorescence staining for collagen I after 1 day of incubation with BBT. (A) Control group and (B) 0.001, (C) 0.01, (D) 0.1 and (E) 1% BBT groups (original magnification, x200; scale bar, 200 μm). BBT, *Bambusa tulda* methanolic extract.

the cells treated with various concentrations of BBT are presented in Figs. 6-9. No marked changes in the expression of collagen I were observed on day 1 (Fig. 6). On day 3, a noticeable increase of collagen I expression was observed following the application of BBT, particularly at a concentration of 1% (Fig. 7). However, the results on day 5 indicated

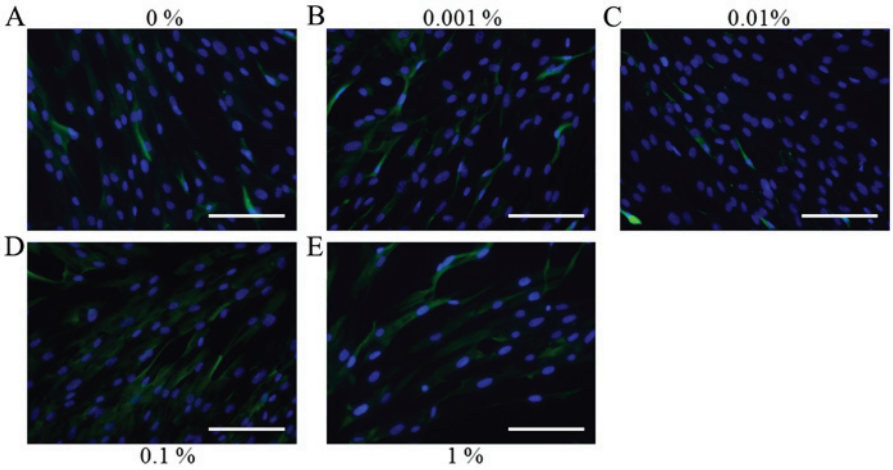

Figure 7. Immunofluorescence staining for collagen I after 3 days of incubation with BBT. (A) Control group and (B) 0.001, (C) 0.01, (D) 0.1 and (E) 1% BBT groups (original magnification, x200; scale bar, 200 μm). BBT, *Bambusa tulda* methanolic extract.

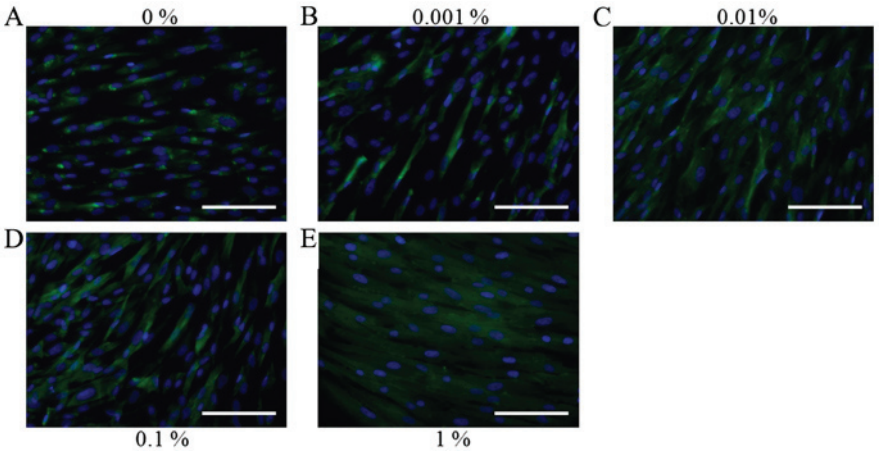

Figure 8. Immunofluorescence staining for collagen I after 5 days of incubation with BBT. (A) Control group and (B) 0.001, (C) 0.01, (D) 0.1 and (E) 1% BBT groups (original magnification, x200; scale bar, 200 μm). BBT, *Bambusa tulda* methanolic extract.

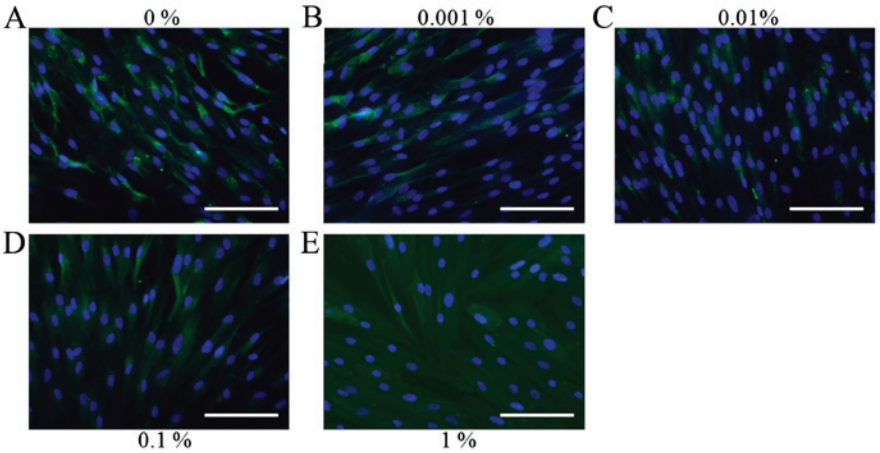

Figure 9. Immunofluorescence staining for collagen I after 7 days of incubation with BBT. (A) Control group and (B) 0.001, (C) 0.01, (D) 0.1 and (E) 1% BBT groups (original magnification, x200; scale bar, 200 μm). BBT, *Bambusa tulda* methanolic extract.

that a gradual reduction of collagen I expression occurred when higher doses of BBT were applied (Fig. 8). Similar trends for collagen I expression were observed in the presence of BBT on day 7 (Fig. 9).

Discussion

The present study investigated the effects of various concentrations of BBT on the morphology and cellular

proliferation of stem cells derived from human gingival tissues. It was clearly demonstrated that the short-term application of *Bambusa tulda* enhanced the proliferation of mesenchymal stem cells with enhanced collagen I expression. Certain herbal extracts, which have long been used in traditional Asian medicine, are used as sources of novel therapeutics (7). These extracts may be considered prevalidated for effectiveness and are expected have fewer safety issues than chemically synthesized drugs (7,8). In previous studies, various herbal extracts have been applied to mesenchymal stem cells for the enhancement of cell proliferation (9). Extracts of another herb, *Polygala tenuifolia*, promoted the proliferation of neural stem cells (10). Similarly, *Ginkgo biloba* herbal extract has been demonstrated to promote osteogenic differentiation of human bone marrow mesenchymal stem cells (11). Promotion of the neuronal differentiation of bone marrow stem cells was achieved by application of *Salvia miltiorrhiza* extract (12). In the present study, application of BBT enhanced the cell proliferation by up to 50% depending on the concentration and incubation time. With this regard, BBT may be applied to overcome the limited number of available cells or improve the survival of implanted stem cells. Herbal extracts may be combined with growth factors for synergistic effects (13).

There has been a great interest in using stem cells in cell therapy due to their promising characteristics that are beneficial for tissue regeneration and treatment of diseases (7). Stem cells have a self-renewal capacity and pluripotency with the ability to differentiate into various cell types, including bone, adipose tissue and cartilage (14,15). Stem cells may be obtained from various sources, including the intraoral area (16,17). Dental pulp and periodontal ligaments are considered preferred sources; however, due to the limited amount of tissue, an additional procedure of tooth extraction is required (6,18). Gingiva may be a more favorable source of stem cells (19). Previous studies by our group indicated that gingiva-derived stem cells possessed colony-forming abilities, plastic adherence and a multilineage differentiation potency with the capacity to undergo osteogensis, adipogenesis and chondrogenesis, and expressed stem cell markers, including CD44, CD73, CD90 and CD105 (6,20). In addition, harvesting stem cells from gingiva may be easier than from bone marrow of the maxilla and mandible due to the lesser invasiveness, pain and paresthesia (21-23).

Various methods have been applied for the extraction of herbs (24). Cold pressing and expeller pressing use a heat-controlled environment, with a higher temperature being applied for expeller pressing (25). Various solvents, including ethanol, methanol, butanol, hexane, methylene chloride, ethyl acetate and water may be used for solvent extraction (7,24,26,27). A novel method of microwave-assisted extraction was also developed for the extraction of *Bambusa bambos* (28). Different solubility and boiling points are noted between different types of solvents (24,29). In a previous study, the dried powdered leaves were extracted with a hydroalcoholic mixture (30). It should be noted that different effects may be achieved depending on the type of solvent and the concentration (24).

Based on these findings, it was concluded that BBT produced beneficial effects on the proliferation of mesenchymal stem cells with enhanced collagen I expression at early time points.

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