*J Osaka Dent Univ* 2023 (April); **57 (1)**: 47−53.

# Aggravation of cellular senescence in human periodontal fibroblasts cultured with tobacco smoke components by stretching stimulation

**Ming Zhao[1], *Jun-ichiro Jo[2], Aki Nishiura[3], Hidetoshi Morikuni[3], Shin-ichi Fujiwara[4], Yoshitomo Honda[5] and Naoyuki Matsumoto[3]**

*[1]Graduate School of Dentistry (Department of Orthodontics), [2]Department of Biomaterials, [3]Department of Orthodontics, [4]Department of Chemistry, [5]Department of Oral Anatomy, Osaka Dental University, 8-1 Kuzuhahanazono-cho, Hirakata-shi, Osaka 573-1121, Japan*
**E-mail: jo-j@cc.osaka-dent.ac.jp*

**Understanding the effect of smoking on oral pathologies at the molecular and cellular level is required for the stable progress of oral medicine. In the present study, we investigated the effect of tobacco smoke components (TSCs) and mechanical stimulation on the senescence of periodontal ligament cells. We cultured human periodontal ligament fibroblasts (HPdLFs) for different durations that were exposed to TSCs collected by our own original procedures. Proliferative activity and cellular senescence after TSC treatment was examined at different points in time. Our results demonstrated that HPdLFs continuously treated with TSCs exhibited characteristics of senescence. Moreover, there was an increased expression of the senescence-related gene when the senescent HPdLFs were cultured with a stretching stimulation. This result clearly suggests that stretching stimulation aggravates cellular senescence. (J Osaka Dent Univ 2023; 57: 47-53)**

**Key words: Cellular senescence; Human periodontal fibroblasts; Tobacco smoke components; Stretching stimulation; In vitro evaluation**

## INTRODUCTION

Smoking is a known cause or exacerbating factor of various diseases.[1, 2] In oral medicine, smoking is known to cause progression of periodontitis and delay of orthodontic tooth movement.[3, 4] It is therefore necessary to understand the effect of smoking on oral pathologies at molecular and cellular levels.

Many studies have examined the biological effect of smoking by analyzing single chemical substances such nicotine in smoke.[5, 6] However, tobacco smoke is composed of more than 5,000 chemical substances including nicotine and tar.[7] Aiming at understanding the biological action of smoking, we developed a method to prepare tobacco smoke components (TSCs) and experimentally demonstrated that TSCs delay orthodontic tooth movement.[8]

To that effect, we focused on the effect of cellular senescence on oral pathologies.[9] Cellular senescence is induced by various stresses inside and outside the body and releases humoral factors called senescence-associated secretory phenotypes (SASPs), which cause damage to surrounding tissues and organs.[10] Based on this knowledge, further studies were conducted regarding the relationship between TSCs and cellular senescence in oral tissue. Periodontal ligament fibroblasts in the periodontal tissue play an important role in the control of oral pathologies.[11] Therefore, we investigated the effect of TSCs on cellular senescence in periodontal ligament fibroblasts. Moreover, because periodontal tissue is constantly subjected to physiological and/or non-physiological forces such as occlusion, cleaning, tapping, and orthodontic treatment, we also examined the influence of mechanical stimulation on the senescent periodontal ligament fibroblasts.

## MATERIALS AND METHODS

### Preparation of TSCs

TSCs were prepared following the procedure previously reported with an apparatus originally designed in the Department of Chemistry, Osaka Dental University.[12] Smoke from 20 cigarettes (Seven Stars, Japan Tobacco, Tokyo, Japan) was channeled into a collection bottle in the apparatus and solubilized with 100 mL of 99.5 vol% ethanol (Fujifilm Wako Pure Chemicals, Osaka, Japan). The solution was then evaporated and re-solubilized with Dulbecco's Modified Eagle Medium (DMEM, Fujifilm Wako Pure Chemicals) to obtain TSCs (50 $\mu$g/mL).

### Cell culture

Normal human periodontal fibroblasts (HPdLF, Walkersville, MD) were cultured in a growth medium, Dulbecco's Modified Eagle Medium (DMEM), containing 10 vol% fetal bovine serum (Hyclone Laboratories, South Logan, UT, USA) and 1 vol% antibiotics (Fujifilm Wako Pure Chemicals) in a humid atmosphere of 5% $CO_2$ at 37°C. The medium was changed every 2 days and passaged every 4 days. HPdLFs with the passage number 6 were used in this study.

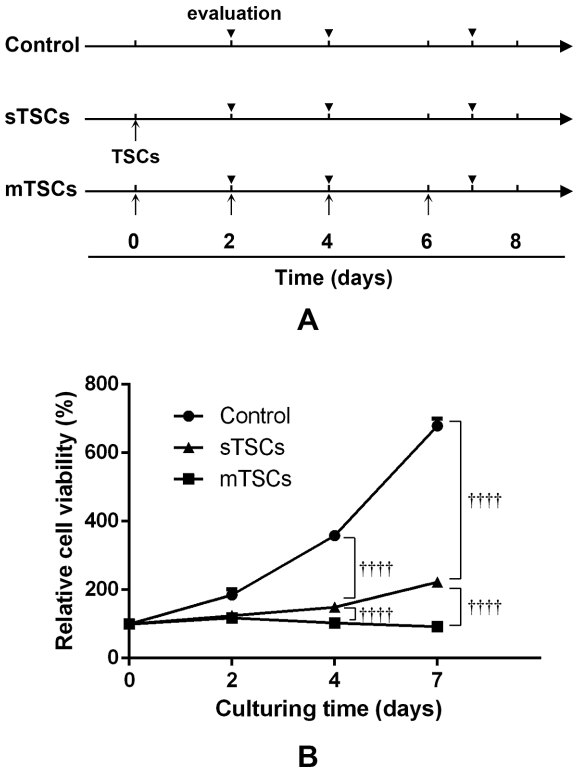


**Fig. 1** Effect of tobacco smoke component treatment on the proliferative activity of human periodontal ligament fibroblasts (HPdLFs). **(A)** Experiment timeline for each group. Arrows (↑) and down-pointing triangles (▽) indicate the timing for changing the medium containing TSCs (50 $\mu$g/mL) and evaluating the cellular viability, respectively. **(B)** Time profiles of HPdLF viability after treatment of each group (●Control, ▲Single TSCs (sTSCs), ■Multiple TSCs (mTSCs), ††††$p < 0.0001$ by ANOVA).

### Cell viability assay

HPdLFs ($2.5 \times 10^4$ cells/well) were seeded in 96-well plates (Thermo Fisher Scientific, Waltham, MA, USA) and cultured in a growth medium for 2 days. After confirming stable cell attachment, cell cultures were started according to the schedule illustrated in Fig. 1 A. In the single TSC (sTSC) group, the cells were cultured with the growth medium containing TSCs (50 $\mu$g/mL) at day 0 and the medium was changed to the growth medium without TSCs every 2 days (days 2, 4 and 6). In the multiple TSC (mTSC) group, the cells were continuously cultured with the growth medium containing TSCs while performing a medium change every 2 days. In the control group, cells were cultured with a normal growth medium without TSCs. Viability of cells at days 2, 4 and 7 in each group was evaluated using Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions.

### Identification of cellular senescence

In an 8-well slide chamber (WATSON, Kobe, Japan), HPdLFs ($5.0 \times 10^4$ cells/well) were seeded and cultured using the same procedures and schedule for the multiple TSCs and control groups listed in Fig. 1 A. Follow-up evaluations for cellular senescence were performed on days 2, 4 and 6.

Senescence-associated $\beta$-galactosidase (SA-$\beta$-gal) activity was evaluated using the cellular senescence detection kit-SPiDER-$\beta$Gal (Dojindo Laboratories) following the manufacturer's instructions. Bafilomycin A 1 working solution was added and incubated in a 5% $CO_2$ incubator at 37°C for 1 hr.

Then, SPiDER-βGal working solution containing 1 μg of Hoechst 33342 (Dojindo Laboratories) was added and placed in a 5% $CO_2$ incubator at 37°C for 30 min. The solution was aspirated and the chamber was washed with phosphate-buffered saline (PBS).

Immunofluorescent staining for γH2AX was performed following the manufacturer's instructions using the DNA damage detection kit-γH2AX (Dojindo Laboratories). Cells were fixed with 4% paraformaldehyde for 15 min at room temperature and permeabilized with 0.25% Triton X-100/PBS solution for 5 min, followed by blocking with 1% bovine serum albumin (BSA; Sigma-Aldrich, Burlington, MA, USA). After adding the anti-γH2AX antibody to the cells, secondary antibodies conjugated with green fluorescent were added and the slide was mounted with DAPI-Fluoromount-G® (Southern Biotech, Birmingham, AL, USA).

For p16 and p21 immunofluorescent staining, cells were fixed in 4% paraformaldehyde for 15 min at room temperature and permeabilized with 0.3% Triton X-100 in PBS buffer for 5 min. Thereafter, cells were incubated with anti-p16-INK 4 A antibody (1 : 50, Proteintech Group, Rosemont, IL, USA) or anti-p21 antibody (1 : 100, Proteintech Group) for 1 hr at 37°C. After adding the anti-Goat IgG antibody conjugated with Alexa Fluor® 488 (1 : 300, Thermo Fisher Scientific), the slide was mounted with DAPI-Fluoromount-G®.

Sample observation was carried out using confocal laser microscopy (LSM-700; Zeiss Microscopy, Jena, Germany) for SA-β-gal activity or fluorescent microscopy (BZ-9000; Keyence, Osaka, Japan) for γH2AX, p21, and p16 expression. All images obtained were counted using Image J ver. 1.51 j 8 (National Institutes of Health, Bethesda, MD, USA) and the percentage of positive cells was calculated.

Quantitative polymerase chain reaction (qPCR) analysis was performed for cells ($1.0 \times 10^5$ cells/well) cultured in a 24-well plate (Thermo Fisher Scientific). Total RNA was extracted from the cells at days 2 and 7 using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Reverse transcription was then performed using a SuperScript™ VILO™ cDNA Synthesis Kit (Thermo Fisher Scientific). Real time qPCR was performed on a StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific) with a solution including TaqMan™ Fast Advanced Master Mix (Thermo Fisher Scientific), TaqMan™ Gene Expression Assay (Thermo Fisher Scientific) for p16 (CDKN2A, Hs00923894_m1) and p21 (CDKN1A, Hs00355782_m1), and cDNA was obtained as above. The PCR conditions were as follows: at 95°C for 10 min, followed by 45 cycles of denaturation (at 95°C for 15 sec) and annealing (at 60°C for 60 sec). A comparative threshold cycle (Ct) method was used to calculate the level of mRNA expression.

**Effect of stretching stimulation on cellular senescence**

To investigate the effect of stretching stimulation on cellular senescence, 6 experimental groups were set according to the schedule illustrated in Fig. 3 A. HPdLFs ($2.5 \times 10^4$ cells/well) were seeded in 4-well stretch chambers (15 × 15 × 10 mm, STREX, Osaka, Japan) and coated with fibronectin (Merck KGaA, Darmstadt, Germany). For the stretching culture, the chamber with cells was treated on an automated cell stretching system (STREX) and intermittently stretched at a strain of 20% and frequency of 10 times/min for 2 days.

After finishing the culture, real time PCR was performed following the same procedures described above using primers for human receptor activator of nuclear factor-kappa B ligand (RANKL, Sino Biological, Kanagawa, Japan), osteoprotegerin (OPG; Sino Biological), p21 (Thermo Fisher Scientific), p 16 (Thermo Fisher Scientific), tumor necrosis factor (TNF)-α (Sino Biological), and interleukin (IL)-6 (Sino Biological).

**Statistical analysis**

Statistical analyses were performed using Microsoft Excel software statistics package (version 2209). Statistical significance was assessed by one-way analysis of variance (ANOVA), with Dunnett's test and Tukey-Kramer test, and by Student's t-test. Graphs were prepared in GraphPad Prism (version

6.01).

## RESULTS

### Continuous exposure of TSCs inhibited the proliferative activity of HPdLFs

The viability of cells treated by different procedures was evaluated to investigate the effect of TSC treatment on the proliferative activity of HPdLFs *in vitro* (Fig. 1). Cell viability was suppressed by TSC while the extent of suppression was influenced by the frequency of exposure to TSCs. In the sTSC group, cell viability was temporally suppressed and later returned to normal after removal. Meanwhile, cell viability in the mTSC group was constantly suppressed during the TSC treatment. These results clearly indicate that continuous use of TSCs results in long-term inhibition of proliferative activity of HPdLFs.

### Continuous exposure to mTSCs induced senescence in HPdLFs

Several senescence-related proteins were fluorescently visualized to investigate whether continuous treatment with TSCs induces senescence in HPdLFs (Fig. 2 A). HPdLFs continuously exposed to mTSCs possessed the activity of SA-$\beta$-gal and expressed the senescence-related proteins $\gamma$H2AX, p21 and p16. The percentages of cells expressing these markers that were continuously exposed to mTSCs was significantly higher than for those not exposed to TSCs. The expression level of p21 rapidly increased after TSC treatment while that of p16 gradually increased. A similar tendency was observed in the expression level of these genes (Fig. 2 B). These results demonstrate that continuous exposure to mTSCs induces senescence in HPdLFs.

### Stretching stimulation increased senescence in HPdLFs

We evaluated the change in gene expression level in senescent cells using the stretch culture in order to explore the effect of stretch stimulation on cellular senescence (Fig. 3). The expression levels of both RANKL and OPG genes decreased upon continuous exposure to mTSCs (Fig. 3 A). The expression level of RANKL genes in cells under stretch conditions was significantly lower than those under normal conditions. In contrast, no change in OPG gene expression levels was observed on stretching stimulation. When evaluated, the RANKL / OPG value decreased on stretching stimulation. The expression level of both p21 and p16 genes increased upon continuous exposure to mTSCs and further increased on stretching stimulation (Fig. 3 B). The expression level of both TNF-$\alpha$ and IL-6 genes also increased following continuous exposure to TSCs and stretching stimulation (Fig. 3 C). These results demonstrate that stretching stimulation increased senescence in HPdLFs.

## DISCUSSION

The present study reveals that a continuous treatment of mTSCs induces cellular senescence in HPdLFs and that stretching stimulation further increases cellular senescence. Several studies have demonstrated that smoking causes cellular senescence in periodontal tissue cells.[13] However, there are no reports examining the effect of tobacco components on senescence in periodontal ligament fibroblasts. Moreover, no study has evaluated the influence of mechanical stimulation on cellular senescence. To the best of our knowledge, this is the first study investigating the effect of the simultaneous action of tobacco components and mechanical stimulation on the senescence of periodontal ligament fibroblasts.

HPdLFs continuously exposed to mTSCs had characteristics of cellular senescence such as an inhibited proliferation (Fig. 1), increased SA-$\beta$-gal activity, and increased expression of senescence-related proteins and genes (Fig. 2). It is well known that cellular senescence is induced through signal pathways of ataxia telangiectasia mutated (ATM)-p53-p21 and p16-retinoblastoma protein (pRb).[14] Based on these results, it is likely that some molecules in TSCs are involved in triggering these pathways. Nicotine is one of the most comparable candidate molecules. Our previous study revealed that TSCs contain 7.9 w/w% nicotine.[12] A preliminary ex-

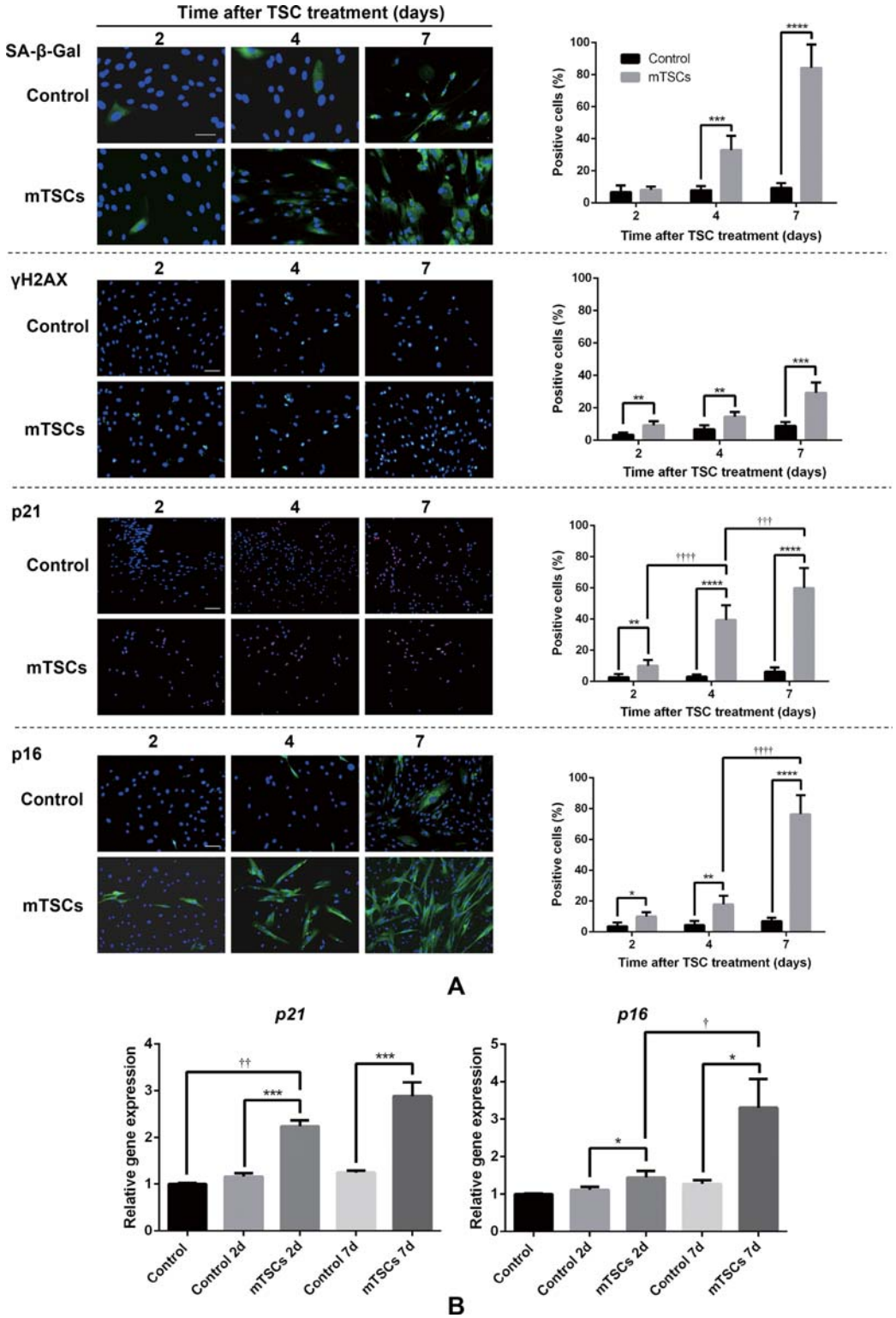


**Fig. 2** Identification of HPdLF senescence after TSC treatment. The experiment timeline of the control or mTSCs groups is the same as that for the viability experiment (Fig. 1 A). **(A)** Representative fluorescent staining of cellular senescence markers (SA-$\beta$-gal, $\gamma$H2AX, p21 and p16) for HPdLF at 2, 4 and 7 days after mTSC treatment, and the percentage of positive cells (Bar: 100 $\mu$m). **(B)** Relative expression level of p21 or p16 genes for HPdLF at 2 and 7 days after mTSC treatment (*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ by Student's t-test, †$p<0.05$, ††$p<0.01$, †††$p<0.001$, ††††$p<0.0001$ by ANOVA).

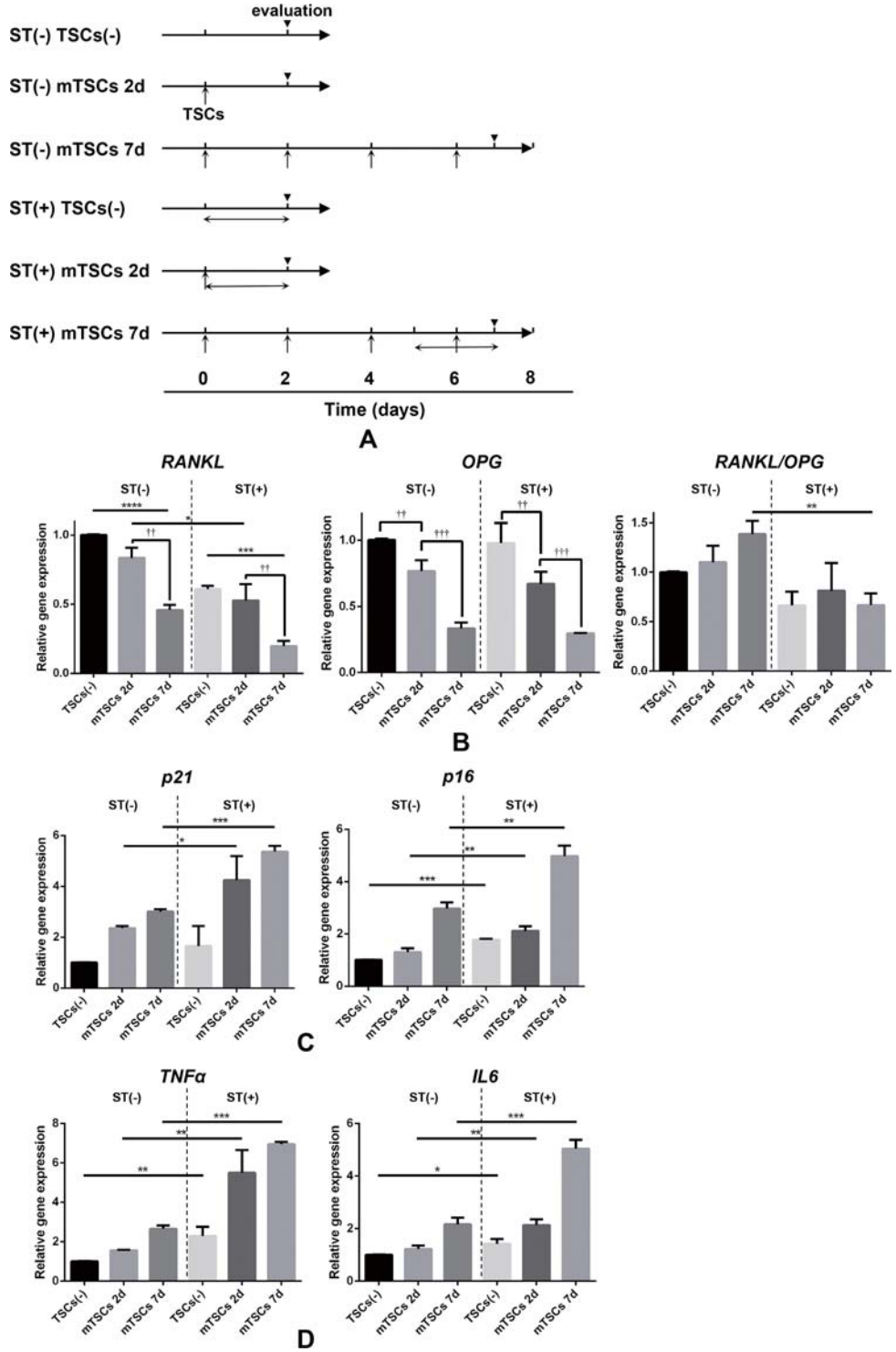


**Fig. 3** Effect of stretching stimulation on the level of gene expression for HPdLF after TSC treatment. **(A)** Experiment timeline of each group. Arrows (↑) and down-pointing triangles (▽) indicate the timing for changing medium containing the TSCs (50 $\mu$g/mL) and for evaluating the gene expression, respectively. In the ST (＋) groups, the stretching stimulation was performed over 2 days indicated by two direction arrows (↔). The expression level **(B)** is shown for bone metabolism-related genes (RANKL, OPG, and RANKL/OPG), **(C)** senescence-related genes (p21 and p16), and **(D)** SASP-related genes (TNF-$\alpha$ and IL-6) for HPdLF after treatment of each group (*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ by Student's t-test, †$p<0.05$, ††$p<0.01$, †††$p<0.001$, ††††$p<0.0001$ by ANOVA).

periment demonstrated that the proliferative activity of HPdLFs continuously exposed to nicotine was similar to that of when exposed to TSCs at the same concentration (data not shown). This result implies that other molecules are involved in cellular senescence. The numerous molecules in TSCs, more than 5,000 molecules, are not identified at present.

Expression level of various genes in the senescent HPdLFs was changed by stretching stimulation (Fig. 3). The ratio of RANKL and OPG genes (RANKL/OPG ratio) tended to decrease after stretching stimulation (Fig. 3 B). This tendency was reported in non-senescent, normal HPdLFs, which agrees with previous studies.[15] However, the reason why the RANKL/OPG ratio decreased by cellular senescence is unclear. In addition, stretching stimulation increased the expression level of senescence-related genes p21 and p16 in both non-senescent and senescent HPdLFs (Fig. 3 C). This result clearly showed that stretching stimulation could be a factor causing cellular senescence, which is consistent with other studies.[16] It is well recognized that cellular senescence induces the production of SASPs. As expected, the expression level of TNF-$\alpha$ and IL-6 genes of SASPs increased on stretching stimulation (Fig. 3 D).

There are two limitations in this study. First, the mechanism of cellular senescence by TSCs is not elucidated. As shown in Fig. 2, p21 was expressed rapidly after mTSCs treatment, while p16 was expressed slowly. This result implies that initial cellular senescence is governed by the activation of the ATM-p53-p21 pathway and that continuous treatment by mTSCs activates the pRb pathway. This point should be clarified in future studies using cell biological techniques. Second, only stretching stimulation was used to investigate the effect of mechanical stimulation on the cellular senescence. Other mechanical stimulations such as compression will help to better understand the relationship between mechanical stimulation and cellular senescence.

## REFERENCES


1. Genco RB, Borgnakke WS. Risk factors for periodontal disease. *Periodontology 2000* 2013; **62**: 59-94.
2. Saha SP, Bhalla DK, Whayne TF Jr, Gairola C. Cigarette smoke and adverse health effects: an overview of research trends and future needs. *Int J Angiol* 2007; **16**: 77-83.
3. Laxman VK, Annaji S. Tobacco use and its effects on the periodontium and periodontal therapy. *J Contemp Dent Pract* 2008; **9**: 97-107.
4. Qin Y, Liu Y, Jiang Y, Mei S, Liu Y, Feng J, Guo L, Du J, Graves DT, Liu Y. Cigarette smoke exposure inhibits osteoclast apoptosis via the mtROS pathway. *J Dent Res* 2021; **100**: 1378-1386.
5. Park GJ, Kim YS, Kang KL, Bae SJ, Baek HS, Auh QS, Chun YH, Park BH, Kim EC. Effects of sirtuin 1 activation on nicotine and lipopolysaccharide-induced cytotoxicity and inflammatory cytokine production in human gingival fibroblasts. *J Periodontal Res* 2013; **48**: 483-492.
6. Zhang W, Song F, Windsor LJ. Effects of tobacco and P. gingivalis on gingival fibroblasts. *J Dent Res* 2010; **89**: 527-531.
7. Talhout R, Schulz T, Florek E, van Benthem J, Wester P, Opperhuizen A. Hazardous compounds in tobacco smoke. *Int J Environ Res Public Health* 2011; **8**: 613-628.
8. Nagaie M, Nishiura A, Honda Y, Fujiwara S, Matsumoto N. A comprehensive mixture of tobacco smoke components retards orthodontic tooth movement via the inhibition of osteoclastogenesis in a rat model. *Int J Mol Sci* 2014; **15**: 18610-18622.
9. Chen S, Zhou D, Liu O, Chen H, Wang Y, Zhou Y. Cellular senescence and periodontitis: mechanisms and therapeutics. *Biology (Basel)* 2022; **11**: 1419.
10. Di Micco R, Krizhanovsky V, Baker D, d'Adda di Fagagna F. Cellular senescence in ageing: from mechanisms to therapeutic opportunities. *Nat Rev Mol Cell Biol* 2021; **22**: 75-95.
11. Boyko GA, Melcher AH, Brunette DM. Formation of new periodontal ligament by periodontal ligament cells implanted *in vivo* after culture *in vitro*. A preliminary study of transplanted roots in the dog. *J Periodontal Res* 1981; **16**: 73-88.
12. Nagaie M, Tsuda S, Makita Y, Nishiura A, Fujiwara S, Matsumoto N. Evaluation of a new scavenging method for the isoloation of cigarette smoke components for use in animal studies. *J Osaka Dent Univ* 2013; **47**: 179-184.
13. Nyunoya T, Monick MM, Klingelhutz A, Yarovinsky TO, Cagley JR, Hunninghake GW. Cigarette smoke induces cellular senescence. *Am J Respir Cell Mol Biol* 2006; **35**: 681-688.
14. Kumari R, Jat P. Mechanisms of cellular senescence: cell cycle arrest and senescence associated secretory phenotype. *Front Cell Dev Biol* 2021; **9**: 645593.
15. Schröder A, Gubernator J, Nazet U, Spanier G, Jantsch J, Proff P, Kirschneck C. Correction to: Effects of sodium chloride on the gene expression profile of periodontal ligament fibroblasts during tensile strain. *J Orofac Orthop* 2022; **83**: 73.
16. Ning L, Gao L, Zhang F, Li X, Wang T. Mechanical stretch induces annulus fibrosus cell senescence through activation of the RhoA/ROCK pathway. *Biomed Res Int* 2021; **2021**: 5321121.