Histol Histopathol (2018) 33: 825-833

http://www.hh.um.es

Histology and Histopathology

From Cell Biology to Tissue Engineering

Combining enamel matrix proteins with mechanical stimuli potentiates human periodontal ligament fibroblasts proliferation and periodontium remodeling

Rui Zou[1,2,3], Wanting Wan[3], Jingjing Li[4], Chanyuan Du[3], Yijie Wang[3], Tian Qian[3] and Lin Niu[1,3]

[1]Key Laboratory of Shaanxi Province for Craniofacial Precision Medicine Research, College of Stomatology, [2]Clinical Research Center of Shaanxi Province for Dental and Maxillofacial Diseases, [3]College of Stomatology, Xi'an Jiaotong University, Xi'an and [4]Taian City Central Hospital, Taian, Shandong, P.R. China

Summary. Background. Collagen I (Col-I) and matrix metalloproteinase-1 (MMP-1) have been implicated in the regeneration and remodeling of the periodontium. Studies have shown that enamel matrix proteins (EMPs) and mechanical stimuli can promote the synthesis and degradation, respectively, of Col-I and MMP-1. However, the effects of the combination of EMPs and mechanical stimuli on human periodontal ligament are not known.

Objective. Our aim was to test the combined effects of EMPs and mechanical stimuli on the proliferation of human periodontal ligament fibroblasts (HPDLFs) and Col-I and MMP-1 mRNA expression.

Methods. Primary HPDLFs were isolated using an enzyme digestion method. To select the optimum EMP concentration and the optimum magnitude and loading time of mechanical stimuli, HPDLFs were stimulated with gradient concentration of EMPs (0 μg/mL, 25 μg/mL, 50 μg/mL, 100 μg/mL and 200 μg/mL) and mechanical stimuli (0 kPa, 25 kPa, 50 kPa, 100 kPa, and 200 kPa for 0 h, 3 h, 6 h, 12 h, and 24 h), respectively. The cell proliferative response was tested by the MTT assay. The impact of EMPs combined with mechanical stimuli on Col-I and MMP-1 mRNA expression were measured by reverse transcription polymerase chain reaction.

Results. 100 μg/mL of EMPs and a 50 kPa mechanical stimulus were chosen as the optimum parameters due to the higher proliferation rates than other doses. The combination of 100 μg/mL of EMPs and a 50 kPa mechanical stimulus significantly stimulated HPDLFs proliferation and increased Col-I and MMP-1 expression levels compared with incubation with two factors alone.

Conclusions. We concluded that the combination of EMPs and mechanical stimulus have synergistic effects on cell growth, cell number, collagen turnover, and periodontium remodeling.

Key words: Human periodontal ligament fibroblasts, Enamel matrix proteins, Mechanical stimuli, Proliferation, Collagen I, Matrix Metalloproteinase-1

Offorint requests to: Dr. Lin Niu, College of Stomatology, Xi'an Jiaotong University, Xi'an, 710004, P.R. China. e-mail: niulin@mail.xjtu.edu.cn

DOI: 10.14670/HH-11-978

Introduction

The periodontal ligament that surrounds the roots and connects them to alveolar bone is an essential component of the periodontium; it consists of fibers, cells, and blood vessels. Human periodontal ligament fibroblasts (HPDLFs) are the most common cell type in the periodontal ligament. These cells can promote the synthesis of collagen by secreting various cytokines, and also degrade collagen by phagocytosis or enzymatic hydrolysis (Berkovitz, 2004). HPDLFs are the effector cells of the periodontal ligament. They play a dominant role in the processes of tissue regeneration, remodeling and damage repair as well as alveolar bone regeneration and repair (Nomura et al., 2012; Minch, 2013). The proliferation of HPDLFs directly reflects the health of the periodontium.

The principal fibers secreted by HPDLFs comprise

the majority of the periodontium. Collagen I (Col-I) is the major component of the principal fibers and accounts for approximately 90 percent of the collagen in the periodontium. Col-I plays a mechanical support role in the periodontium by maintaining the integrity of the periodontal ligament and ensuring the biological functions of other components. Furthermore, Col-I can interact with corresponding receptor integrins on the cell surface through specific amino acid sequences and impacts vital process such as cell proliferation, migration and gene expression. Under normal physiological conditions, collagen fiber turnover is regulated by matrix metalloproteinase (MMP) related genes.

MMPs are a family of endopeptidases that depend upon metal ions such as Zn^{2+} and Ca^{2+}. To date, at least 26 types of human MMPs have been reported; these MMPs are named MMP-1 to MMP-26 based on the chronological order of their discovery. MMPs have the ability to cleave almost all components of the extracellular matrix (ECM) and basement membrane, which is essential for normal physiological processes such as embryonic development, cell migration, wound healing and tissue remodeling (Nagase and Woessner, 1999; Page-McCaw et al., 2007). However, abnormalities in these enzymes play a role in many pathological conditions, such as tumor metastasis and inflammation-induced tissue destruction (Sternlicht and Werb, 2001; Stamenkovic, 2003). MMP activity is regulated by endogenous tissue inhibitors of matrix metalloproteinases (TIMPs) (WojtowiczPraga et al., 1997; Brew et al., 2000). The balance between MMPs and TIMPs is tightly regulated during tissue remodeling and wound healing. MMP activity is necessary during the first step of wound healing to allow the locomotion and arrival of cells and growth factors to the injured area, whereas TIMP activity is essential during the late stage of tissue repair to minimize tissue damage (Trengove et al., 1999; Gill and Parks, 2008).

Mechanical stimuli are common in the oral environment. Mechanical stimuli such as orthodontic forces and occlusal forces can be transmitted from the tooth crowns to the periodontium, and finally, the periodontal ligament will be affected by strain and/or stress stimuli. HPDLFs are the direct effector cells of this mechanical stimuli. Mechanical stimuli within the physiological range can stabilize HPDLFs and even enhance their differentiation and proliferation, which ensures the long-term dynamic balance of regeneration and remodeling in the periodontium. Numerous studies have shown that physiological mechanical stimuli promote regeneration and remodeling by regulating cell proliferation and affecting expression of genes such as Col-I, Col-III MMP-1, MMP-2, MMP-8, and MMP-9 (Howard et al., 1998; Bolcato-Bellemin et al., 2000; Cantarella et al., 2006; El-Awady et al., 2013; Shibata et al., 2014).

EMPs are extracted from the enamel matrix and can induce HPDLFs differentiation (Amin et al., 2013), proliferation, adhesion and migration (Rincon et al., 2005; Lin et al., 2013). EMPs can also up-regulate the expression of many key genes, such as osteocalcin (OCN), Col-I, MMP-1, and MMP-2, and thus promote periodontium healing. The commercially available enamel matrix derivative (EMD; a type of protein derivative) EMDOGAIN® (Suzuki et al., 2014) is in clinical use in parts of Europe and America to treat periodontal disease. *In vivo* it would be unlikely to have EMP and mechanical stimuli independent of each other. However, the effects of the combination of EMPs and mechanical stimulation on HPDLFs function have not been studied. Therefore, the purpose of the present study was to assess the impact of combining EMPs and mechanical stimuli on HPDLFs proliferation and Col-I and MMP-1 expression.

Materials and methods

Ethics statements

Periodontal ligaments were obtained from recently extracted human teeth from 4 individuals 12-16 years of age who were treated at the Stomatology Hospital of Xi'an Jiaotong University. Tooth extractions were performed by researchers of our research team. All teeth were extracted for orthodontic reasons and were healthy with no sign of disease. The participants and their parents or guardians all signed an informed consent form, and they were in agreement with this research. The ethical protocol approved by the Medical Ethics Committee of Stomatology Hospital of Xi'an Jiaotong University College of Medicine (Permit Number: 2016 NO.047) had been obtained.

HPDLFs isolation and culture

To culture primary HPDLFs, we obtained the middle part of the periodontal ligament surrounding the root and rinsed them with PBS three times. These tissues were digested with 0.1% type I collagenase (Sigma, USA) with intermittent shaking for 30 min at 37°C. The digested tissues were inoculated onto Petri dishes with Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) containing 15% fetal calf serum (FBS, Gibco, USA) and 100 U/mL penicillin-streptomycin solution (Solarbio, China), and then incubated in 5% CO_2 at 37°C. The medium was changed every 72 hours. Cells were purified by the enzyme digestion method. Cells from the third passage were characterized by immunofluorescent staining assay (Tang et al., 2016). HPDLFs were fixed in 4% paraformaldehyde for 15 min and permeabilized in 0.1% Triton for 10 min. The cells were blocked with 5% goat serum for 1h and incubated with anti-vimentin antibody (Abcam, UK) or anti-keratin antibody (Abcam, UK) at 4°C overnight. Then a DyLight 488 or a DyLight 594 polyclonal antibody (Zhuangzhibio, China) was used as secondary antibody, respectively. Nuclei were counterstained with DAPI. The cells used for our experiments were from the 3rd-5th

passage and showed relatively stable growth traits.

EMP dosing experiments

Cells were stimulated with increasing EMP concentration: 0 μg/mL (E0), 25 μg/mL (E1), 50 μg/mL (E2), 100 μg/mL (E3) and 200 μg/mL (E4). The HPDLFs were seeded into five (5 different time points) 96-well plates (5 rows and 9 columns each, 5 rows for 5 different EMP concentration) at a density of 2×10^3/well. After 24 h of culture, cells were placed in DMEM containing 1% FBS for an additional 24 h and then different concentration of EMPs (Straumn, Sweden) were applied to cells for 24 h. The culture medium (complete DMEM) was changed every 2 days. Cells were cultured for 0, 1, 3, 5 or 7 days and one plate was selected randomly and 20 μl of MTT and 180 μl of DMEM without fetal calf serum were added. Four hours later, the medium was replaced with 150 μl of DMSO. After 10 min of shaking, the absorbance of the cells in each well was measured at 492 nm with an ELISA plate reader (Amersham, Sweden) to evaluate proliferation (cell viability).

Mechanical stimulus experiments

The controllable cell loading device used in these experiments was designed by the Central Laboratory of the Hospital of Stomatology, Xi'an Jiaotong University. The device consisted of a gas supply, cell sample chamber, pressure and temperature monitors, a pressure regulator and an air decompression modulator (Fig. 1). The device worked as follows: clean gas under high pressure regulated by a pressure regulator flowed into the cell sample chamber at a stable flow rate. When the pressure in the cell sample chamber reached the predetermined value, the pressure monitor automatically shut off the air inlet and formed a stable closed environment. The pressure acted on the cell culture fluid and produced a stable hydraulic pressure upon the cells. After the experiment was completed, the gas flow through the air decompression modulator was halted. The gas contained 5% CO_2, and the temperature was set at 37°C.

The cells were divided into 5 groups depending on the intensity of the mechanical stimulus: 0 kPa (S0), 25 kPa (S1), 50 kPa (S2), 100 kPa (S3) and 200 kPa (S4). Each load, cells were seeded into five (5 different time points) 96-well plates at the same seeding density described above. Nine wells in each plate were used for this experiment. After 24 h of incubation and 24 h of serum deprivation, the cells were placed in fresh culture medium and subjected to intermittent mechanical stimuli using a barometric load cell that provided an appropriate growth environment for the cells. The mechanical stimulus included 0 kPa, 25 kPa, 50 kPa, 100 kPa, and 200 kPa for 0 h, 3 h, 6 h, 12 h, and 24 h. Then, cell proliferation (viability) was tested with the same MTT method. The control group was not subjected to any mechanical stimulus.

Combined mechanical and EMP stimuli

These experiments included four groups: control group (C), mechanical stimulus group (MS), EMP stimulated group (EMP) and combined EMP and

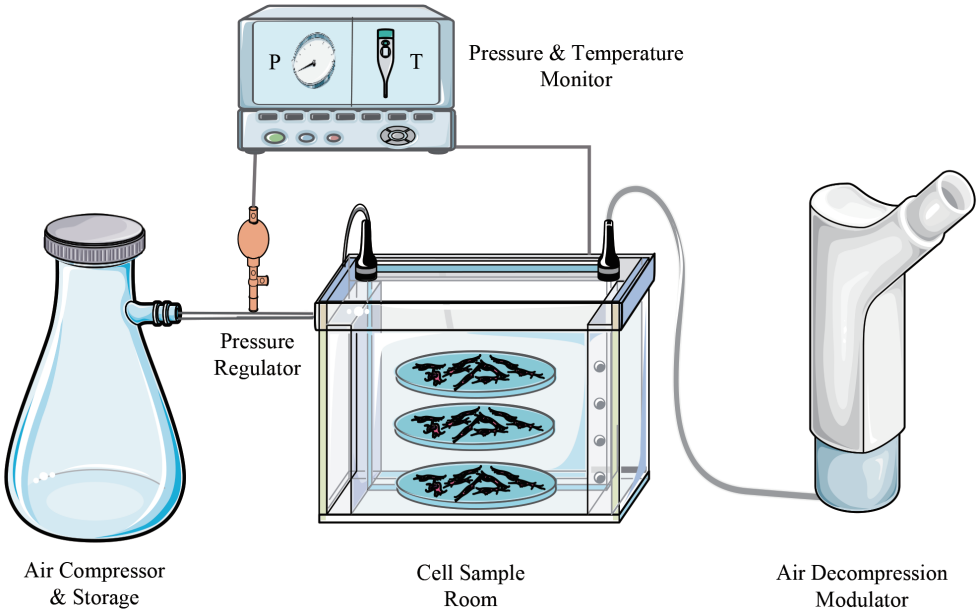

Fig. 1. Schematic of the pressure-loading device.

mechanical stimuli group (EMP+MS). As the control group, C group was not given special treatments. Optimum stress was loaded for the MS group, and the most appropriate EMP concentration was applied to EMP group. Both stimuli were applied simultaneously to EMP+MS. Cells were incubated in four 96-well plates at the same seeding density as above. For the mechanically stimulated groups cells were loaded in the barometric load cell. Incubation times were based on the EMP screening experiments (3 days) and the mechanical stimulus screening experiments (12 h). For the combined group, cells were first given EMP for 3 days and stress was loaded in the last 12h during this 3-day incubation. Cell morphology was observed and proliferation was tested using the MTT method described above.

Real-time PCR analysis

The experimental groups and the stimuli for each group were the same as described above. Total RNA was extracted using TRIzol (Invitrogen, USA) according to the manufacturer's protocol. First-strand cDNA was synthesized using a RevertAid First Strand cDNA Synthesis Kit (Thermo, USA). Then the cDNA was

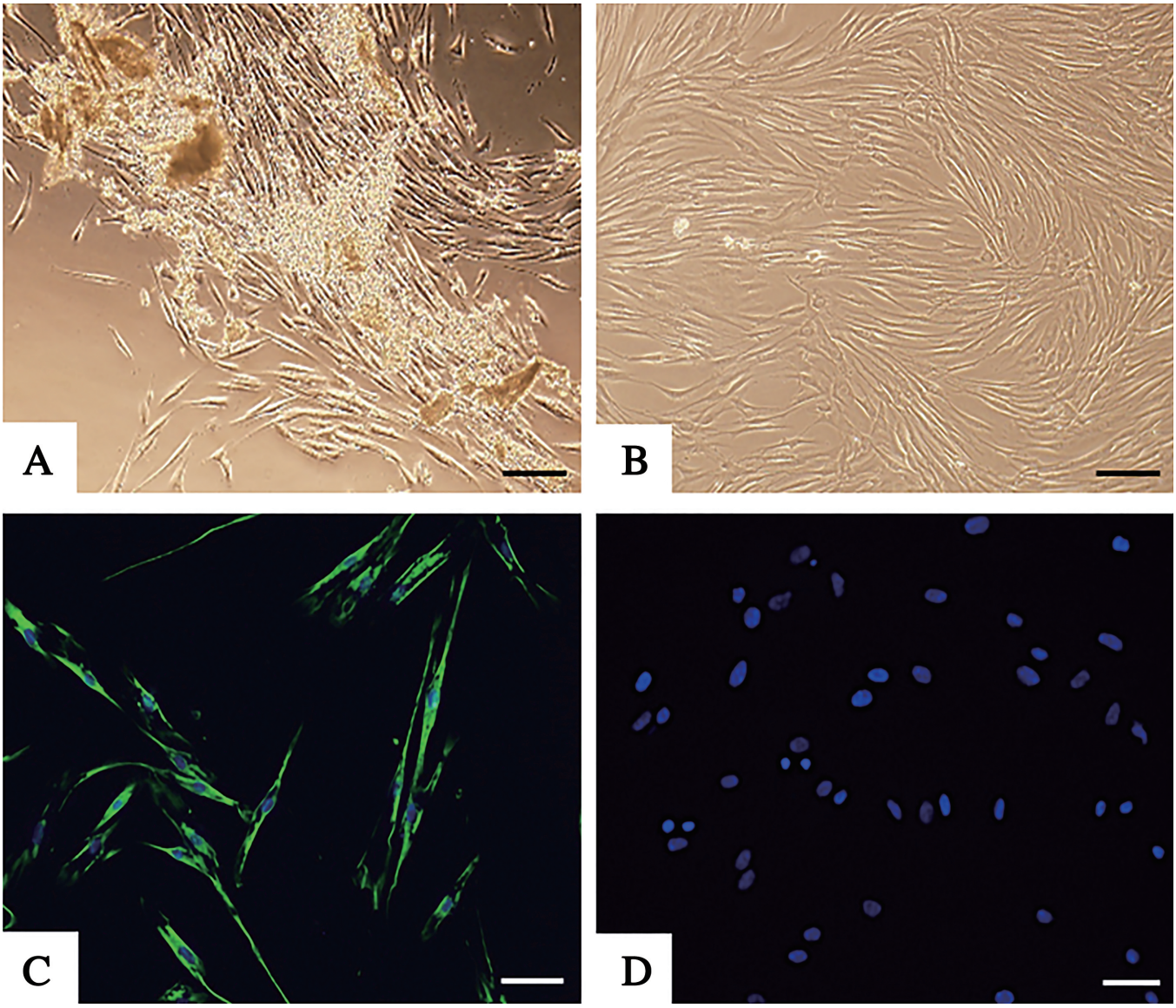

Fig. 2. Characterization of HPDLFs. Primary HPDLFs had grown out of periodontal ligament tissues by days 7 **(A)** and the 3rd passage HPDLFs exhibited a long fusiform morphology **(B)**. Immunofluorescent staining of HPDLFs were anti-vimentin positive **(C)** (nucleus, blue; vimentin, green) and anti-cytokeratin negative **(D)** (nucleus, blue). Scale bars: A, B, 161 μm; C, D, 64 μm.

mixed with SYBR Green PCR Master Mix (Life Technologies, USA). PCR was performed for 30-35 cycle at 95°C for 15 s, 85-90°C for 15 s and 75°C for 30 s in a DNA thermal cycler (Eppendorf, Germany). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The primer sequences of Col-I, MMP-1 and GAPDH were designed (Table 1).

PCRs were repeated on at least three independent RNA preparations. The Col-I mRNA and MMP-1 mRNA expression values were calculated using the $2^{-\Delta\Delta Ct}$ method (ΔCt = the mean cycle threshold Ct of the target gene – the mean Ct of GAPDH; ΔΔCt = ΔCt of

Table 1. Sequence of primers for RT-PCR.

Gene	Forward primer	Reverse primer
Col-I	CCTGGATGCCATCAAAGTCT	GAATCCATCGGTCATGCTCT
MMP-1	GGGAGATCATCGGGACAACTC	GGGCCTGGTTGAAAAGCAT
GAPDH	ACCCACTCCTCCACCTTTG	CACCACCCTGTTGCTGTAG

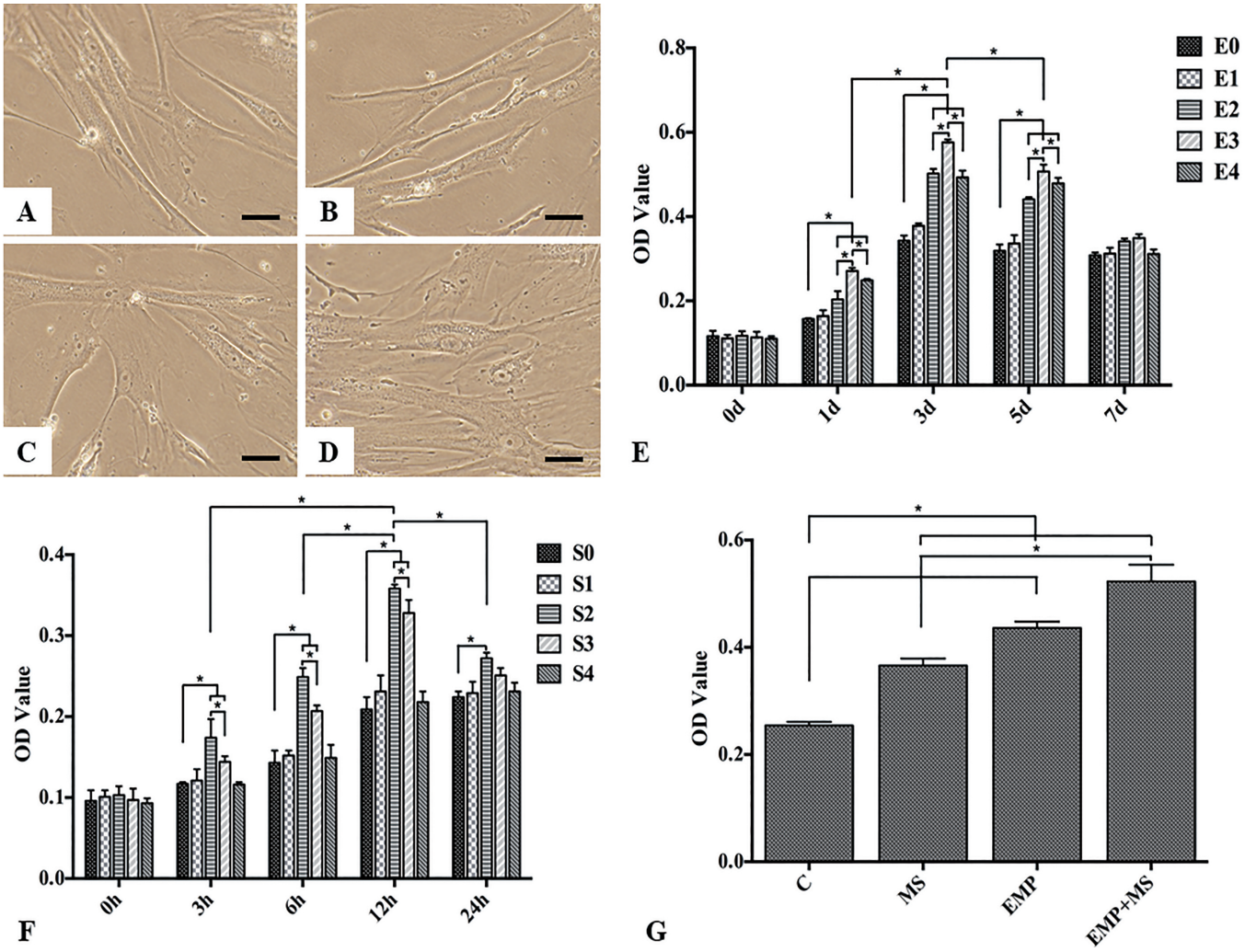

Fig. 3. Effects of stimuli on HPDLFs morphology and proliferation. HPDLFs morphology of C group **(A)**, EMP group **(B)**, MS group **(C)** and EMP+ MS group **(D)** were observed under microscope. **E.** HPDLFs proliferation (viability) was determined with the MTT assay with increasing concentrations of EMP: 0 μg/mL(E0), 25 μg/mL(E1), 50 μg/mL(E2), 100 μg/mL(E3), and 200 μg/mL(E4) of EMPs. **F.** Cells were loaded with increasing intensity of the mechanical stimuli: 0 kPa (S0), 25 kPa (S1), 50 kPa (S2), 100 kPa (S3), 200 kPa (S4). Comparing to S0, HPDLFs at S2 presented the highest proliferation at 12h. **G.** When EMP and mechanical stimuli combined, HPDLFs of EMP+MS presented the highest proliferation compared to other groups. Data is presented as means ± SD. Symbol * indicates significant differences; $p<0.05$. Scale bars: 52 μm.

experimental group -ΔCt of control group).

Statistical analysis

The data were analyzed by one-way analysis of variance with SPSS13.0. The values are expressed as the mean ± standard deviation (SD). P<0.05 was considered statistically significant.

Results

Characterization of HPDLFs

Primary HPDLFs had grown out of periodontal ligament tissues by days 7 (Fig. 2A) and the 3rd passage HPDLFs exhibited a long fusiform morphology when observed by an inverted phase contrast microscopy (Fig. 2B). Immunofluorescent staining of HPDLFs were anti-vimentin (the mesenchymal marker) positive (Fig. 2C) and anti-cytokeratin negative (the epithelial marker) (Fig. 2D).

EMP dosing experiments

As shown in Fig. 3E, the HPDLFs in the E2, E3 and E4 groups presented higher proliferation at the 1 d, 3 d, and 5 d time points compared with the E0 group (P<0.05). With respect to the EMP concentration, the HPDLFs in group E3 presented significantly higher proliferation rates than the E2 and E4 groups (P<0.05). With respect to time, HPDLFs in E3 had significantly higher proliferation rates on day 3 than day 1 or 5 (P<0.05). Therefore, we selected 100 μg/mL as the optimum concentration for use in all subsequent experiments.

Mechanical stimuli dosing experiments

As shown in Fig. 3F, HPDLFs in groups S2 and S3 presented higher proliferation rates at 3 h, 6 h, and 12 h compared with S0 (P<0.05). With respect to the magnitude of the mechanical stimulus, HPDLFs in S2 presented significantly higher proliferation than S3 (P<0.05). With respect to time, HPDLFs in S2 presented the highest proliferation rates at 12 h compared to other times (P<0.05). Therefore, we selected 50 kPa as the most appropriate mechanical stimulus and 12 h as the optimum time for subequent experiments.

Combined mechanical and EMP stimuli

The HPDLFs in groups C and EMP exhibited a long fusiform morphology (Fig. 3A,B) while cells in group MS and EMP+MS began to present a few branches (Fig. 3C,D). Cells in groups MS, EMP and EMP+MS presented higher proliferation rates as compared to C (P<0.05). Moreover, the proliferation of EMP+MS was significantly higher than the proliferation of MS and EMP (P<0.05) (Fig. 3G).

The effects of combined mechanical stimuli and EMPs on Col-I and MMP-1 mRNA expression

MS, EMP and EMP+MS groups had higher Col-I and MMP-1 mRNA expression levels compared with C (P<0.05), and Col-I and MMP-1 mRNA expression

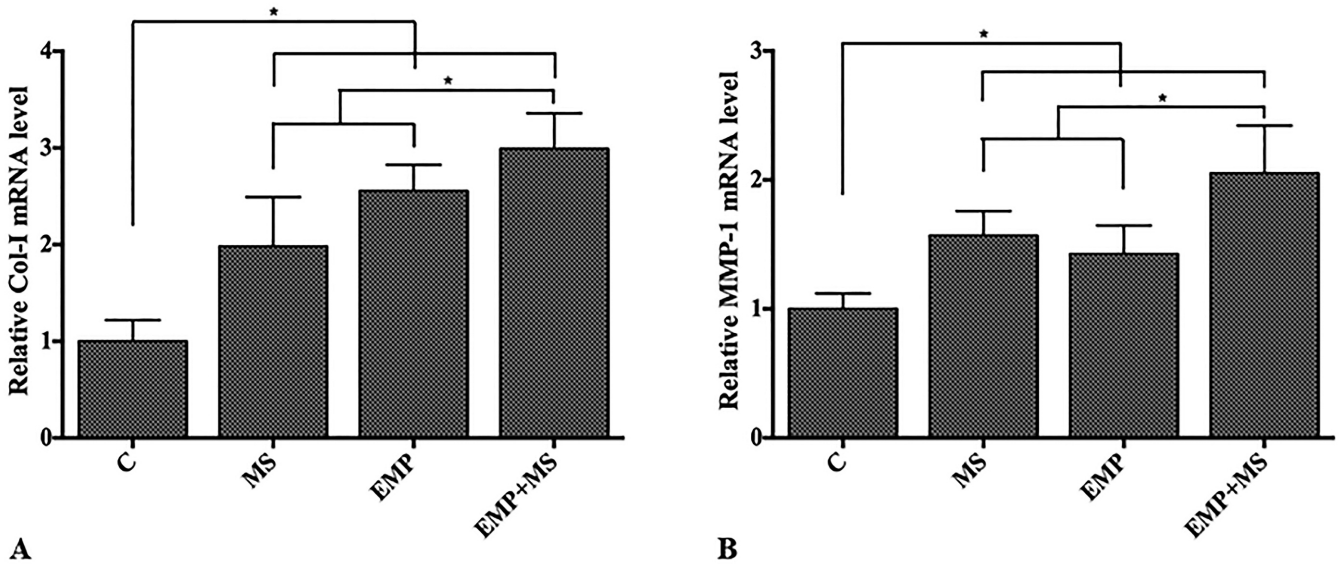

Fig. 4. Combined effects of EMPs and mechanical stimuli on Col-I and MMP-1 mRNA expression. Col-I **(A)** and MMP-1 **(B)** mRNA levels were measured by quantitative real-time PCR data are presented as the means ± SD of C, MS, EMP and EMP+MS and expressed as a percentage of the mRNA level. Data is presented as means ± SD. Symbol * indicates significant differences; p<0.05.

levels were significantly higher in EMP+MS than in MS and EMP ($P<0.05$) (Fig. 4A,B).

Discussion

In this study, we extracted HPDLFs from periodontium and then tested the cellular effects of combining two agonists, EMPs and mechanical stimuli. Our results show that the combination of these two stimuli had synergistic effects on HPDLFs proliferation and Col-I and MMP-1 expression.

HPDLFs are the major cells in the periodontal ligament, although some other cells (e.g. Malasesz epithelia, osteoblast) also exist. After being purified by subculture, these extracted cells had typical long fusiform morphology of fibroblast. As one of the most important markers of HPDLF, vimentin, which is also a structural component of the cytoskeleton, indicates a mesenchymal origin of the cells. The presence of vimentin is characteristic for fibroblasts and distinguishes them from epithelial cells and endothelial cells (Staszyk and Gasse, 2007). And the negative expression of cytokeratin, an important protein in epithelial cells, also helps indicate cell origins.

A number of EMP biological effects have been previously reported. Amin et al. (2013) found that 100 mg/L was the optimum EMP concentration for increasing HPDLFs differentiation capacity. Schlueter et al. (2007), reported that EMPs promoted angiogenesis and HPDLFs proliferation. Xiao et al. (2008) found that EMPs at 100 mg/L, 150 mg/L and 200 mg/L stimulated Col-I synthesis at 5 d. Our own results show that an EMP concentration of 100 μg/mL significantly up-regulated HPDLFs proliferation compared to the other concentrations on days 1, 3 and 5. Thus, we used EMP at 100 μg/mL for the experiments involving mechanical stimuli.

Cells in intact tissues are exposed to a number of mechanical stimuli of varying intensities (Fujihara et al., 2010). The main mechanical stimuli to which HPDLFs are exposed to are the orthodontic and occlusal forces transmitted from the tooth crowns to the periodontium (Li et al., 2013). Various mechanical loading devices have been used to stimulate cells *in vitro*. The loading parameters used by researchers (i.e., the magnitude, waveform, frequency, cycle number and duration of loading) differ to a large extent according to the researchers' preferences, leading to different results (Kook and Lee, 2012). Some studies have suggested that the use of mechanical stimuli that result in 9-18% cell deformation is preferred and that the duration of loading should be 0.5-5 d (Sato et al., 1990). According to Fan et al. (2012), HPDLFs are more sensitive to stress than strain. Although the *in vitro* loading parameters cannot completely simulate *in vivo* conditions, the finding that mechanical stimuli can promote HPDLFs proliferation is seen consistently. Yousefian et al. (1995) hypothesized that the periodontal ligament space was a continuous hydrostatic system and that forces applied to this environment by means of mastication or orthodontic appliances should create hydrostatic pressure. Their study showed that HPDLFs proliferation increased significantly at 24 and 48 hours following stimulation of these cells with −30 gm/cm2 of negative hydrostatic pressure. According to the study of Wang et al. (2005), moderate forces (l000 μstrain) significantly promoted HPDLFs proliferation, whereas excessive force (3000 μstrain) suppressed it. The proliferation curve presented an initial slow rise (2 h), followed by rapid growth (2-4 h), and then a stationary period (4-6 h). In addition to proliferation, Bolcato-Bellemin et al. (2000) found that MMP-1 mRNA expression was increased with the stimuli of 20 kPa and 12 h force loading. Loading parameters vary a lot in relevant studies (Bolcato-Bellemin et al., 2000; Wang et al., 2013; Zhang et al., 2016), and the differences may result from the use of different experimental setups and conditions. Based on prior studies, we chose hydrostatic pressure and used 0 kPa, 25 kPa, 50 kPa, 100 kPa and 200 kPa and 0 h, 3 h, 6 h, 12 h and 24 h loading times for our experiments. Our study showed that 50 kPa and 12 h of loading optimally promoted HPDLF proliferation. Thus, these conditions were then chosen as the optimum loading parameters for subsequent experiments.

Cellular self-renewal and proliferation are the foundations of periodontal regeneration and are dependent on cell number. Several published studies have shown that depending on both the magnitude of mechanical stimulation and EMP concentrations these signals can promote proliferation, differentiation and migration of HPDLFs and up-regulate the expression of many related genes. However, the combined effects of mechanical stimulation and EMPs on the periodontium have not been previously reported. We report not only on the effects of mechanical stimulation and EMPs but also on the synergistic effects of the combination of these two stimuli on HPDLFs proliferation and remodeling.

According to our data, the ability of the combination of EMPs and mechanical stimuli to promote cell proliferation and up-regulate Col-I and MMP-1 mRNA was greater than that by EMPs alone. Thus, two factors, EMPs and mechanical stimuli, play a positive feedback effect on periodontal reconstruction. Whereas, Nokhbehsaim's results demonstrated that biomechanical loading antagonized significantly the EMD-induced effects in PDL cells (Nokhbehsaim et al., 2011). This contradiction maybe results from the fact that the cells in their experiment received strain, instead of stress in our research. Besides, other MMPs and TIMPs will be evaluated in our next research in consideration of their importance in periodontium remodeling. As EMDOGAIN® has been put into clinical use in periodontal disease treatment, we could add some proper mechanical stimuli (orthodontic force, occlusal force, etc.) to enhance periodontium regeneration. However, the magnitude and the mode of this specific force in clinic remain to be explored in further studies.

The EMPs family consists of various proteins and

growth factors; thus, the biological mechanism is also complex. The previously accepted view was that EMPs functioned primarily by promoting transcription and expression of IGF-I and TGF-β in HPDLCs. However, recent studies have shown that multiple signal transduction pathways are activated by EMPs, including the G protein-coupled receptor signaling pathway (Kemoun et al., 2011). Further studies are needed to clarify what signaling pathways are activated during periodontal regeneration.

In summary, we report that mechanical stimuli and EMPs activate complimentary signaling pathways which are synergistic when applied concurrently. The limitation of our study is that the cytomechanical device used only applied pressure rather than the complex mechanical signals seen with occlusal forces. Whether further refining the mechanical stimuli alters the biological response remains to be seen. EMPs are already in clinical use in parts of Europe and America with good results. However, our study suggests that addition of an occlusal force/signal may provide further clinical benefit.

Acknowledgements. This work was supported by the National Natural Science Foundation of China (No: 81300915), the Natural Science Basic Research Plan in Shaanxi province of China (No: 2015JM8414) and the Fundamental Research Funds for the Central Universities, China (No: xjj2016129).

References

Amin H.D., Olsen I., Knowles J.C., Dard M. and Donos N. (2013). Effects of enamel matrix proteins on multi-lineage differentiation of periodontal ligament cells *in vitro*. Acta Biomater. 9, 4796-4805.

Berkovitz B.K. (2004). Periodontal ligament: structural and clinical correlates. Dent. Update 31, 46-54.

Bolcato-Bellemin A.L., Elkaim R., Abehsera A., Fausser J.L., Haikel Y. and Tenenbaum H. (2000). Expression of mRNAs encoding for alpha and beta integrin subunits, MMPs, and TIMPs in stretched human periodontal ligament and gingival fibroblasts. J. Dent. Res. 79, 1712-1716.

Brew K., Dinakarpandian D. and Nagase H. (2000), Tissue inhibitors of metalloproteinases: evolution, structure and function. Biochim. Biophys. Acta 1477, 267-283.

Cantarella G., Cantarella R., Caltabiano M., Risuglia N., Bernardini R. and Leonardi R. (2006). Levels of matrix metalloproteinases 1 and 2 in human gingival crevicular fluid during initial tooth movement. Am. J. Orthod. Dentofacial Orthop. 130, 568.e11-16.

El-Awady A.R., Lapp C.A., Gamal A.Y., Sharawy M.M., Wenger K.H., Cutler C.W. and Messer R.L.W. (2013). Human periodontal ligament fibroblast responses to compression in chronic periodontitis. J. Clin. Periodontol. 40, 661-671.

Fan X., Wang Y., Li Y. and Zhoo Z. (2012). Early proliferation changes and differences of gene expression in human periodontal ligament fibroblasts subjected to tensile and compressive stress. Hua xi kou qiang yi xue za zhi. 30, 463-473.

Fujihara C., Yamada S., Ozaki N., Takeshita N., Kawaki H., Takano-Yamamoto T. and Murakami S. (2010). Role of mechanical stress-induced glutamate signaling-associated molecules in cytodifferentiation of periodontal ligament cells. J. Biol. Chem. 285, 28286-28297.

Gill S.E. and Parks W.C. (2008). Metalloproteinases and their inhibitors: regulators of wound healing, Int. J. Biochem. Cell Biol. 40, 1334-1347.

Howard P.S., Kucich U., Taliwal R. and Korostoff J.M. (1998). Mechanical forces alter extracellular matrix synthesis by human periodontal ligament fibroblasts. J. Periodontal Res. 33, 500-508.

Kemoun P., Gronthos S., Snead M.L., Rue J., Courtois B., Vaysse F., Salles J.P. and Brunel G. (2011). The role of cell surface markers and enamel matrix derivatives on human periodontal ligament mesenchymal progenitor responses *in vitro*. Biomaterials 32, 7375-7388.

Kook S.H. and Lee J.C. (2012). Tensile force inhibits the proliferation of human periodontal ligament fibroblasts through Ras-p38 MAPK up-regulation. J. Cell. Physiol. 227, 1098-1106.

Li L., Han M., Li S., Wang L. and Xu Y. (2013). Cyclic tensile stress during physiological occlusal force enhances osteogenic differentiation of human periodontal ligament cells via ERK1/2-Elk1 MAPK pathway. DNA Cell Biol. 32, 488-497.

Lin Z.K., Shu R., Cheng L., Song Z.C., Guo Q.M. and Zhang X.L. (2013). Effects of rhAm on attachment, proliferation and immigration of human fibroblasts. Shanghai kou qiang yi xue. 22, 7-12.

Minch L. (2013). Material properties of periodontal ligaments. Postepy Hig. Med. Dosw. 67, 1261-1264.

Nagase H. and Woessner J.F. (1999). Matrix metalloproteinases. J. Biol. Chem. 274, 21491-21494.

Nokhbehsaim M., Deschner B., Bourauel C., Reimann S., Winter J., Rath B., Jäger A., Jepsen S. and Deschner J. (2011). Interactions of enamel matrix derivative and biomechanical loading in periodontal regenerative healing. J. Periodontol. 82, 1725-1734.

Nomura Y., Ishikawa M., Yashiro Y., Sanggarnjanavanich S., Yamaguchi T., Arai C., Noda K., Takano Y., Nakamura Y. and Hanada N. (2012). Human periodontal ligament fibroblasts are the optimal cell source for induced pluripotent stem cells. Histochem. Cell Biol. 137, 719-732.

Page-McCaw A., Ewald A.J. and Werb Z. (2007). Matrix metalloproteinases and the regulation of tissue remodeling. Nat. Rev. Mol. Cell Biol. 8, 221-233.

Rincon J.C., Xiao Y., Young W.G. and Bartold P.M. (2005). Enhanced proliferation, attachment and osteopontin expression by porcine periodontal cells exposed to Emdogain. Arch. Oral Biol. 50, 1047-1054.

Sato M., Theret D.P., Wheeler L.T., Ohshima N. and Nerem R.M. (1990). Application of the micropipette technique to the measurement of cultured porcine aortic endothelial cell viscoelastic properties. J. Biomech. Eng. 112, 263-268.

Schlueter S.R., Carnes D.L. and Cochran D.L. (2007). *In vitro* effects of enamel matrix derivative on microvascular cells. J. Periodontol. 78, 141-151.

Shibata M., Shintaku Y., Matsuzaki K. and Uematsu S. (2014). The effect of IL-17 on the production of proinflammatory cytokines and matrix metalloproteinase-1 by human periodontal ligament fibroblasts. Orthod. Craniofac. Res. 17, 60-68.

Stamenkovic I. (2003). Extracellular matrix remodelling: the role of matrix metalloproteinases. J. Pathol. 200, 448-464.

Staszyk C and Gasse H. (2007). Primary culture of fibroblasts and

cementoblasts of the equine periondontium. Res. Vet. Sci. 82, 150-157.

Sternlicht M.D. and Werb Z. (2001). How matrix metalloproteinases regulate cell behavior. Annu. Rev. Cell. Dev. Biol. 17, 463-516.

Suzuki R., Nemoto E. and Shimauchi H. (2014). Cyclic tensile force up-regulates BMP-2 expression through MAP kinase and COX-2/PGE2 signaling pathways in human periodontal ligament cells. Exp. Cell Res. 323, 232-241.

Tang H.N., Xia Y., Yu Y., Wu R.X., Gao L.N. and Chen F.M. (2016). Stem cells derived from "inflamed" and healthy periodontal ligament tissues and their sheet functionalities: a patient-matched comparison. J. Clin. Periodontol. 43, 72-84.

Trengove N.J., Stacey M.C., Macauley S., Bennett N., Gibson J., Burslem F., Murphy G. and Schultz G. (1999). Analysis of the acute and chronic wound environments: the role of proteases and their inhibitors. Wound Repair Regen. 7, 442-452.

Wang Y., Jia Y., Zhao Z.H. and Zhang Z.P. (2005). Effects of mechanical stress on the proliferation kinetics of the human periodontal fibroblast cells *in vitro*. Hua xi kou qiang yi xue za zhi. 23, 332-334.

Wang Y., Wang J., Bai D., Song J., Ye R., Zhao Z., Lei L., Hao J., Jiang C., Fang S., An S., Cheng Q. and Li J. (2013). Cell proliferation is promoted by compressive stress during early stage of chondrogenic differentiation of rat BMSCs. J. Cell Physiol. 228, 1935-1942.

WojtowiczPraga S.M., Dickson R.B. and Hawkins M.J. (1997). Matrix metalloproteinase inhibitors. Invest. New Drugs 15, 61-75.

Xiao B., Guo S., Pan Y. and Liu D. (2008). Effects of enamel matrix proteins on attachment, proliferation and pre-mRNA of type I collagen synthesis of human dermal fibroblast cells *in vitro*. Zhongguo xiu fu chong jian wai ke za zhi. 22, 1113-1116.

Yousefian J., Firouzian F., Shanfeld J., Ngan P., Lanese R. and Davidovitch Z. (1995). A new experimental model for studying the response of periodontal ligament cells to hydrostatic pressure. Am. J. Orthod. Dentofacial Orthop. 108, 402-409.

Zhang L., Liu W., Zhao J., Ma X., Shen L., Zhang Y., Jin F. and Jin Y. (2016). Mechanical stress regulates osteogenic differentiation and RANKL/OPG ratio in periodontal ligament stem cells by the Wnt/β-catenin pathway. Biochim. Biophys. Acta 1860, 2211-2219.

Accepted February 27, 2018