

## Effects of Equiaxial Strain on the Differentiation of Dental Pulp Stem Cells without Using Biochemical Reagents

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**Abstract:** During orthodontic treatments, applied mechanical forces create strain and result in tooth movement through the alveolar bone. This response to mechanical strain is a fundamental biological reaction. The present study evaluated the effect of equiaxial strain within the range of orthodontic forces on the osteogenic differentiation of human dental pulp stem cells (hDPSCs). Following isolation and culture of hDPSCs, 3<sup>rd</sup> passage cells were transferred on a silicone membrane covered with collagen. Cell adhesion to the membrane was evaluated under scanning electron microscope (SEM). Cells were divided into three groups: the first group was placed in a conventional culture medium, transferred to an equiaxial stretching device (3% strain for 2 weeks). The positive control was placed in an osteogenic medium with no mechanical strain. The negative control group was placed in the conventional culture medium with no mechanical strain either. Study groups were evaluated for expression of osteogenic markers (Alkaline phosphatase and Osteopontin) with immunofluorescence and real time PCR. SEM images revealed optimal adhesion of cells to the silicone membrane. Immunofluorescence study demonstrated that osteocalcin expression occurred after 2 weeks in the two groups under mechanical and chemical signals. After application of equiaxial strain, level of expression of osteogenic markers was significantly higher than in the negative and positive control groups. Based on the study results, static equiaxial strain which mimics the types of orthodontic forces can result in differentiation of hDPSCs to osteoblasts. The results obtained may be used in cell therapy and tissue engineering.

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## **1 Introduction**

Orthodontic forces are transferred to the periodontal ligament and eventually to the alveolar bone through the teeth. As the result of orthodontic force application, alveolar bone resorption occurs at the pressure side while osteogenesis takes place at the tension side during tooth movement [1]. Orthodontic tooth movements (OTM) are the result of biological reactions to external mechanical stimuli [2].

At present, cellular mechanotransduction and the mechanism by which cells convert mechanical signals into biochemical responses have attracted lots of attention [3]. Although the exact mechanism of osteogenesis in orthodontics has not been thoroughly evaluated, several mechanisms i.e. mechanotransduction have been suggested for interpretation of the conversion of mechanical signals to cellular responses and subsequent alteration in cell function [4]. Numerous studies have evaluated mechanotransduction through membrane ion channels and integrins when mechanical forces are exerted on cells via the extracellular matrix [5]. In order to explain cellular responses in this respect, various cell lines have been studied. Dental pulp stem cells (DPSC) are a cellular reservoir for different fields of tissue engineering. These cells are isolated from human dental pulp and proliferated in the laboratory [6]. DPSCs just like other somatic stem cells have shown capability to differentiate into various cell types under *in vitro* and *in-vivo* conditions [7]. Several studies have demonstrated the differentiation of DPSCs to osteoblasts in presence of certain biochemical reagents [8-10]. Presently, these cells can be isolated from deciduous or even decayed teeth [11-12] and can be used as a reservoir of stem cells for various purposes.

More knowledge about the cascade of molecular events that occur during orthodontic tooth movement can undoubtedly result in a more efficient treatment with a lower risk of unfavorable outcomes [13]. In previous studies on mechanical stretch, uniaxial strain was mainly used on different cell types [14-16]. Laboratory studies have shown that mechanical strain can lead to differentiation of bone marrow [17] and periodontal ligament stem cells [18]. However, the effect of equiaxial strain on DPSCs has not yet been evaluated. Wang and Thampatty in their review study on cell mechanobiology mentioned the importance of further investigations to determine cellular mechanisms in response to various types of strain [19]. Thus, considering the static equiaxial nature of force exerted by orthodontic appliances, the present study aimed at evaluating the changes in different bone biomarkers in DPSCs following the application of equiaxial strain under *in-vitro* conditions.

## **2 Materials and Methods**

All cell culture chemicals and supplies were purchased from Sigma (NY, USA) and Gibco-BRL (Grand Island, NY, USA) unless otherwise noted.

### **2.1 Isolation and culture of DPSCs:**

After obtaining patients' consent, impacted 3<sup>rd</sup> molars from one donors (Male 25 years) after extraction were placed in phosphate buffered saline (PBS) solution containing antibiotics and transferred to the reference laboratory of cell and molecular oral biology. Dental pulp was carefully extracted from the teeth. Pulp tissue was digested by a solution containing collagenase I (3 mg/ml) and dispase (4 mg/ml) for 30 to 60 minutes at 37°C. After centrifugation at 800 g for 10 minutes, the prepared cell suspension was transferred to a flask containing Dulbecco's Modified Eagle's Medium (DMEM), Fetal Bovine Serum (FBS) (20%) and antibiotic (1%) and incubated at 37°C, 5% CO<sub>2</sub> and 95% moisture. The culture medium was refreshed every 3 days. Cells were passaged after proliferation and covering approximately 70% of the flask surface using trypsin enzyme. The 3<sup>th</sup> passage cells were used for examinations.

### **2.2 Membrane preparation:**

In this study, a medical grade silicone membrane was used. A circle was cut out of the membrane with 5cm diameter in order to be placed in the equiaxial device. To enhance cell adhesion, membrane surface was covered with 0.5 mg/ml type I collagen (Sigma) in 0.2% citric acid. After washing with PBS, the membrane was exposed to UV radiation for 30 minutes and then cell suspension in an amount of  $1 \times 10^6$  cells in 700 microliter culture medium was transferred to the membrane and placed in an incubator for 24 hours.

### **2.3 SEM preparation:**

After 24 hours, cells were fixed in glutaraldehyde 2.5% for 2 hours in a refrigerator. Samples were air dried after rinsing with buffer and dehydration using alcohol of different percentages. Cell-containing membranes were assessed under scanning electron microscope (SEM) at 24 kV (KYKY-EM3200, China).

### **2.4 Static equiaxial strain:**

In order to apply mechanical strain to human dental pulp stem cells cultured on a silicone membrane, an equiaxial strain device designed in the National Cell Bank of Iran was used (Fig. 1). This device has two main components of mechanical and

electrical part. The motor movement is transferred through a ball screw to a collection of parts. The movement of this collection of parts eventually creates strain in silicone membrane. The container placed over the membrane is made of 316 L stainless steel alloy which is biocompatible. The membrane along with attached superior and inferior parts can be separated from the device; thus, when working under the hood, there is no need to move the entire device under the hood. The applied strain was equiaxial, static and with a magnitude of 3% which was continuously ex-

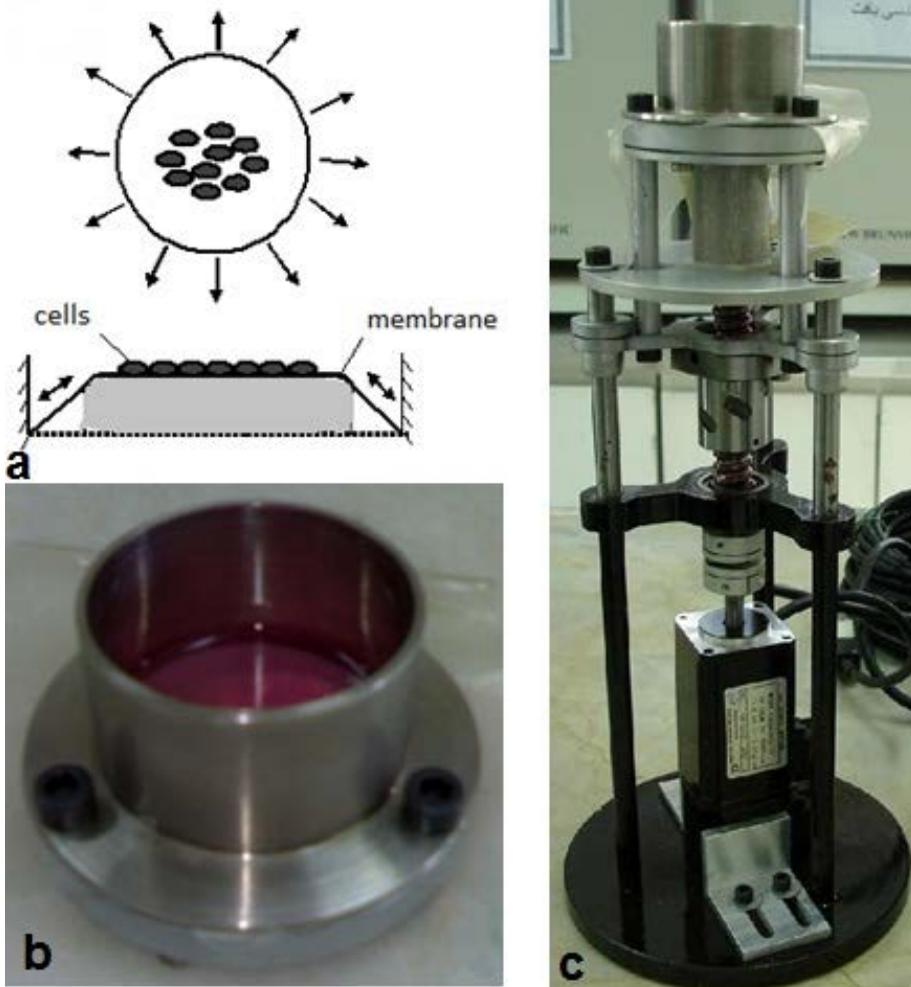


Figure 1: Schematic view of the method of applying equiaxial tensile strain (a), superior view of the actual device (b) and lateral view of the device (c).

erted on cells for 2 weeks. Conventional culture medium (DMEM, 10% FBS, 1% antibiotic/antimycotic) was used. Positive and negative control groups did not receive any strain and placed on a silicone membrane in cell culture plate containing osteogenic medium (Fresh medium contains DMEM and 10% FBS supplemented with 50  $\mu\text{M}$  ascorbate-2 phosphate (Sigma, NY, USA), 10 mM  $\beta$ -glycerophosphate (Sigma, NY, USA) and 0.1  $\mu\text{M}$  dexamethasone) or conventional medium (DMEM, 10% FBS, 1% antibiotic/antimycotic). The medium used for cells cultured on the scaffold was refreshed after 24 hours or the cells were transferred to the device.

### **2.5 Assessment of osteocalcin expression by fluorescent immunocytochemistry:**

In order to determine the stimulation of osteogenic differentiation in response to tensile strain, expression of osteocalcin in cells was tested using immunofluorescence [20]. Cells in all 3 groups were tested and positive and negative controls were washed with PBS and fixed with paraformaldehyde 4% for 30 minutes at 4°C. After irrigation, nonspecific antibodies were blocked with 0.5% goat serum for one hour and the samples were incubated with osteocalcin primary antibodies (polyclonal rabbit anti-bovine with cross-reactivity to human, 1:50 dilution, Chemi-Con, Temecula, CA) overnight at 4°C followed by incubation with secondary antibodies (anti-mouse IgG-FITC 1:160 dilution; Sigma, St. Louis, MO) for 3 hours. In the final step, samples were washed with PBS and photographed using fluorescent microscope.

### **2.6 Real-time PCR:**

After 2 weeks of exertion of the mentioned equiaxial strain, silicon membrane was washed with PBS (phosphate buffer saline). Then total RNA of the cells was extracted using RNeasy plus Mini Kit (QIAGEN, MD, USA) according to the manufacturer's instructions. The purity and concentration of extracted RNA was measured by nanophotometer (Implen, Germany). RNA samples with high qualities (absorbance 260/280nm  $\geq 1.8$ ) and concentration of 400 ng and more are acceptable and cDNA synthesis was carried out using QuantiTect Reverse Transcription Kit (QIAGEN, MD, USA).

SYBR Green Real-time PCR was used for quantitative analysis of osteopontin and ALPL expression. Reactions were performed on ABI StepOne system using StepOne v2.1 software with SYBR Green PCR master mix (Applied Biosystems, Foster City, USA). The following thermal cycling profile was used: holding stage set at 95°C (10 min); cycling stage set at temperatures varying from 95°C (15 sec) and 60°C (1 min) and run for 40 cycles; and melt curve stage set at 95°C (15 sec), 60°C (1 min) and 95°C (15 sec). Each sample was assessed in triplicate for each gene of interest and mean  $C_T$  (comparative threshold cycle) of triplicate reactions

for each gene was applied in data analysis.

Primer Express v3.0 software (Applied Biosystems) was utilized for designing primers. All primers which recommended by primer express were analyzed with gene runner to analysis their sequences. RPL13A (ribosomal protein large subunit 13a) was selected as housekeeping gene in this study. Alkaline phosphatase is an early differentiation marker and Osteopontin is expressed in the late stage of osteogenic differentiation. Osteopontin and ALPL values were normalized to RPL13a expression as endogenous control.

The sequence of RPL13A, osteopontin (OPN) and ALPL primers was as follows:

RPL13A: Forward primer= 5'-CCTGGAGGAGAAGAGGAAAGAGA-3', Reverse primer= 5'-TTGAGGACCTCTGTGTATTTGTCAA-3'. OPN: Forward primer= 5'-AACGCCGACCAAGGAAAACCT-3', Reverse primer= 5'-GGCCACAGCATCTGGGTATT-3'. ALPL: Forward primer= 5'-CCTGGACCTCGTTGACACCT-3', Reverse primer= 5'-GTCCCCTGGCTCGAAGAGA-3'

### 2.7 Statistical analysis:

SPSS version 16.0 software was used for statistical analysis. All samples were averaged and the means for each group were compared using one way analysis of variance (ANOVA) to determine statistically significant differences between the three groups. Values were reported as mean  $\pm$  standard deviation for the individual groups.  $P < 0.05$  was considered statistically significant.

## 3 Results

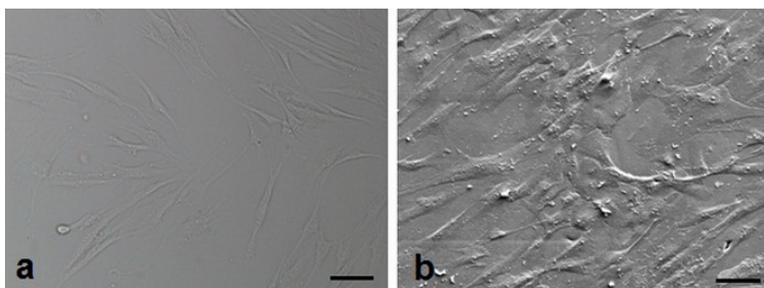


Figure 2: Picture of DPSCs on the silicone membrane under inverted microscope (scale bar=150  $\mu\text{m}$ ) (a) and SEM (scale bar=100  $\mu\text{m}$ ) (b).

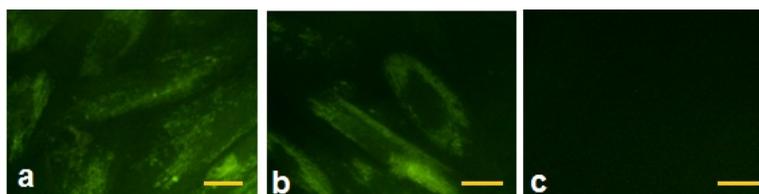


Figure 3: Immunofluorescence staining of osteocalcin marker in the test group under mechanical signals (a), positive control group (b) and negative control group (c) (scale bar=50  $\mu$ m).

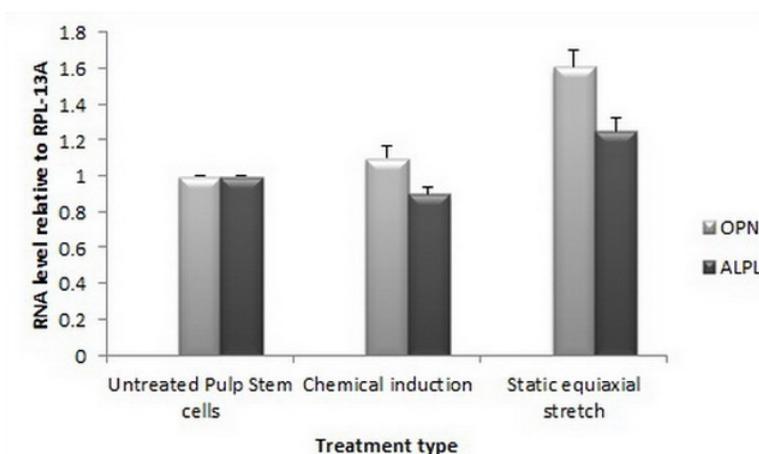


Figure 4: Comparison between the 3 groups: negative control, chemical induction (positive control) and equiaxial strain. The mRNA levels were normalized relative to RPL-13A as reference gene.  $n = 3$  (triplicates per group).  $P < 0.05$  relative to control groups.

In order to ensure adequate cell adhesion to the membrane covered with collagen, cells were evaluated 24 hours after their placement on the membrane with SEM. Images in both inverted and scanning electron microscopes were indicative of adequate cell adhesion to the membrane (Fig. 2).

Immunofluorescence study demonstrated that osteocalcin expression occurred after 2 weeks in the 2 groups under mechanical and chemical signals but such expression did not occur in the negative control group (Fig. 3).

Osteogenic differentiation of human dental pulp stem cells was also evaluated quantitatively in the three study groups using real-time PCR. Fig.4 presents the level of expression of markers in the three study groups.

#### **4 Discussion**

Studies have indicated that following the application of orthodontic forces, at one side (pressure side) collagen fibers are relaxed and unloaded leading to the unloading of the alveolar bone and resulting in its resorption; whereas, in the opposite side (tension side), stretching of collagen fibers in the periodontal ligament results in positive strain which subsequently leads to osteogenesis. The strain depends on the material properties of the periodontal ligament and the magnitude of applied force [1]. Since the nature of orthodontic forces is mostly static and several studies have demonstrated the positive effects of orthodontic forces [21], the present study aimed at testing the hypothesis that equiaxial mechanical strain without the presence of osteogenic medium can result in differentiation of DPSCs.

Adhesion of cells to the substrate is among the main steps in evaluation of the effect of mechanical stimuli on cells. Efficient cell adhesion to the substrate especially under mechanical stimuli initiates a cascade of continuous events. On the contrary, if adequate attachment of cells to the substrate does not occur, upon the exertion of mechanical force, cells are detached and cell population is lost. In many studies, type I collagen or fibronectin are used for covering the surface of silicone membrane to enhance the adhesion of cells to the surface [22-24]. In the present study, membrane surface was also covered with collagen and SEM indicated appropriate adhesion of cells to the membrane.

Laboratory examinations have revealed that mechanical force application leads to increased expression of bone morphogenetic protein (BMP)-2 in bone marrow stem cells as well as expression of osteocalcin, osteopontin and other markers in periodontal ligament cells (PDLs) [17-18]. Also, DPSCs under hydrostatic pressure have demonstrated increased expression of BMP-2 [25]. The present study evaluated the expression of osteopontin and Alkaline phosphatase (ALPL) markers. These two markers are indicative of the differentiation of stem cells to osteoblasts and play an important role in bone remodeling [26]. Quantitative RT-PCR showed that the highest level of expression of osteopontin and other related genes was observed in the test group under mechanical strain after 2 weeks. Expression of OPN and ALPL increased 60% and 30% in comparison with untreated stem cells respectively. Level of expression of the mentioned two markers was relatively lower in the group in osteogenic medium. The present study sought to assess the effect of equiaxial strain without the presence of osteogenic medium on DPSCs. The results showed favorable differentiation of cells to osteoblasts in the test group compared to the samples in osteogenic medium. Immunofluorescence results showed the effect of equiaxial strain on the DPSCs differentiation too. Jang et al. evaluated the combined effects of surface morphology and mechanical straining magnitudes on the differentiation of mesenchymal stem cells without using biochemical reagents

and concluded that mechanical signals without the use of chemical reagents can result in cell differentiation as well [22].

Results of different studies on DPSCs have been controversial. Some researchers have reported that mechanical stress causes odontoblastic/osteoblastic differentiation of DPSCs [27] while some others have demonstrated that mechanical forces have no significant effect on DPSCs [28]. On the other hand, Cai et al, in their study reported that uniaxial tensile strain inhibits osteogenic differentiation of DPSCs [14]. The existing controversies about the effects of mechanical strain are probably attributed to the different designs of laboratory studies and also the difference in type of force, duration of force application and its static or dynamic nature and thus, the obtained results are usually not comparable. Additionally, when studying the orthodontic forces, it should be kept in mind that different types of tooth movements result in different distribution of forces.

The present study had several limitations: (1) only one type of force was tested on cells, (2) gene expression was only evaluated 2 weeks after the culture and thus, the effect of time on mRNA expression was not assessed, (3) expression of type I collagen was not evaluated. Since the membrane surface was covered with collagen, it was difficult to distinguish between the newly synthesized collagen and the existing collagen covering the membrane surface. (4) In laboratory studies on the effect of orthodontic forces on stem cells, it would be preferred to use a scaffold similar to the alveolar bone hard tissue. (5) This study only evaluated the effect of static force on cells while recent studies on orthodontics and bone biology have pointed that cyclic forces yield faster bone remodeling [29]. These limitations have to be eliminated in future investigations on this subject.

## 5 Conclusion

In summary, the present study results demonstrated that equiaxial static tensile strain (3%) leads to increased expression of osteopontin and some other markers in DPSCs (in the absence of osteogenic factors) and expression level of these factors was higher in the test group compared to positive and negative control groups. The current study enhanced our knowledge about the mechanical conditioning of stem cells. This study was the first to assess the effect of equiaxial strain on differentiation of DPSCs.

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