The Effect Of Light Compressive And Tensile Orthodontics Forces On Sclerostin And Periostin Expressions In Human Periodontal Ligament Cells: An Invitro Study

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Research Article

Keywords: Sclerostin, Periostin, Orthodontic Tooth Movement, Gene Expression

Posted Date: April 28th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1423451/v1

Abstract

Background

Orthodontic treatment induces bone resorption on the compression side while on the tension side stimulates bone formation. The process involves a complex interaction of signalling molecules. The aim of the present study is to assess the expression of key regulatory proteins associated with bone remodeling, cell proliferation and apoptosis in response to different types of orthodontic forces at varied time points in periodontal ligament (PDL) cells.

Materials and methods and Results

PDL cells were isolated from extracted teeth and were divided into two groups based on the type and time of applied forces: Group 1 exposed to compressive forces for 0, 6, 24, 48, 72 hours and group 2 imposed to equi-axial tension forces in 1Hz frequency and 10% elongation in 0, 24, 48, 72 hours. Real-Time Quantitative Reverse Transcription PCR (qRT-PCR) was applied to evaluate the gene expression. The secretion of Sclerostin and Periostin was assessed using Enzyme-Linked Immunosorbent Assay (ELISA).

4',6-diamidino-2-phenylindole staining (DAPI) was used to evaluate apoptosis.

The expression of Sclerostin(SOST) elevated at protein and gene levels under compression force while the application of tensile force induced the expression of Periostin(POSTN), Runt-related transcription factor 2(RUNX2) and reduced SOST expression in a time-dependent manner. Proliferating cell nuclear antigen (PCNA), the marker of proliferation increased under tensile force. The compression forces induced apoptosis and increased gene expression of apoptotic regulators, Caspase9, and B-cell lymphoma 2(BCL2).

Conclusion

The periostin and sclerostin play important role in orthodontic loads and their expressions under compressive and tensile forces are affected by both variables of time and type of applied forces. Application of compressive stress increases apoptosis while tensile force induces cell proliferation.

Introduction

During orthodontic force the bone resorption is dominant in the side undergoing compressive forces whereas at the tensile side the bone formation is occuring. Despite many researches, the exact mechanism of orthodontic tooth movement is still unknown and the process is associated with some challenges. Through application of mechanical pressure, the tooth root approaches the alveolar bone wall resulting in reduced local blood flow that can lead to cell death and formation of the necrotic cell-free area called hyalinized tissue regarded as an undesirable effect of tooth movement.[1] In order to achieve a better understanding of the impact of orthodontic mechanics on tooth movement and avoid

subsequent side effects, it is essential to understand the molecular response of periodontal cells to different orthodontic mechanical forces.

Applying tooth movement forces affect many biological process in the tensile and compression area including bone formation and resorption that all involves interaction of different pathways and signalling molecules. SOSTand POSTN are important regulators of bone homeostasis. The SOST gene is responsible for encoding the 22kDa sclerostin protein, which is mainly secreted by osteocytes and hypertrophic chondrocytes.[2] It is known as the antagonist of the Wnt pathway that inhibits bone formation through binding to the Wnt co-receptor Low-density lipoprotein receptor protein5/6(LRP5 / 6) and preventing downstream signaling pathway.[3] The periostin is a 90 kDa extracellular protein secreted from fibroblasts present in the periodontal ligament and periosteum.[4–6] It was shown that in the absence of periostin, alveolar bone loss increase due to the induction of osteoclastic activity.[7] Indeed, periostin has a regulatory role on bone formation through direct stimulation of Wnt/b-catenin signaling pathway and an indirect inhibitory role on SOST expression.[8] RUNX2 is a transcription factor that regulates sclerostin expression via binding to SOST promoter.[9] It was shown that silencing of POSTN can decrease the expression of RUNX2 and there is a synergistic relationship between POSTN and RUNX2 in osteoblastic differentiation.[10]

Programmed cell death, known as apoptosis, plays an important role in the establishment of homeostasis in the process of development and disease. The intrinsic pathway of cell death is triggered by internal stimuli such as DNA damage, oxidative stress, and hypoxia leading to the destruction of the outer mitochondrial membrane and the release of cytochrome C into the cytoplasm of the caspase9 complex, resulting in the formation of apoptosome. The apoptosome activates caspase3, which in turn activates cytoplasmic endonuclease and other caspases leading to chromatin condensation and eventually cells degradation.[11, 12] During orthodontic tooth movements, the incidence of hypoxia increase that might result in the apoptosis of compressed periodontal ligament cells. On the other hand, the hypoxia induces the release of hypoxia-inducible factor-1α that could escalate the osteogenic differentiation in PDL cells.[13] This knowledge indicate the presence of complex dynamic process in orthodontic tooth movement involves signaling molecules related to apoptosis, bone formation and resorption. Evaluating the expression profile of regulatory molecules involved in orthodontic bone formation and resorption will provide a better and broader view of the key factors affecting orthodontic tooth movement that could also help us to improve the efficiency and speed of dental movements as well as reducing treatment complications.

Materials And Methods

Isolation and culture of periodontal ligament cells

The periodontally healthy and non-carious first premolars were collected from young patients without any metabolic and systemic disease referred to the specialized dental clinic of Tehran University of Medical Sciences (TUMS) for orthodontic treatment after receiving informed consent forms. The protocol used in

the present study was examined and approved by the Ethics Committee of the Vice Chancellor for Research and Technology of Tehran University of Medical Sciences (IR.TUMS.VCR.REC.1398.480). After being extracted, teeth were immersed in Hank solution containing 1% penicillin / streptomycin and amphotericin B (25 µg / mL) and were rapidly transferred to the Dentistry Research Institute of TUMS.

After rinsing in PBS buffer, periodontal tissue was scraped from the middle third of the tooth root surface followed by 20 minutes incubation in dispase (4 mg/ml) and type 1 collagenase (3 mg / ml) at 37 °C. After 5 min centrifugation (500g), primary cells were transferred to the culture plates and incubated in α-MEM (Cerogen, Darmstadt,Germany) containing 10% fetal bovine serum, 1% penicillin / streptomycin (BioSera, Paris,France), and 1% L-glutamine with 5% CO2 at 37 °C. In this study, cells at fifth to seventh passages were used.

Applying tensile forces

The cells were cultured on a 250 µm-thick Poly DimethylSiloxane (PDMS) (Sylgards184, Dow Corning, Midland, MICH, USA) membrane and examined in a Custom Built machine (Pasteur Institute of Iran, Tehran) under equiaxial tensile stresses as described in detail previously.[14] In order to ensure greater adhesion of cells to silicones surface, PDMS membrane was incubated in 0.2% gelatin for 20 minutes before culturing the cells.

The cells at 80% confluency underwent forces with a frequency of 1 Hz and 10% elongation (strain) for 24, 48, and 72 hours. After each time period, the culture medium was collected and stored at 20°C for ELISA testing.

Applying compressive forces

In the compressive model, cells were cultured on a glass slide (38×25 ×0.2 mm). The silicone membrane of the same size was then placed on glass slides. A total of 25 gr force (25 gr/cm^2) was applied on silicone membrane using 240 gr lead granules, each weighing 1 gr. The experiments were performed at intervals of 6, 24, 48, and 72 hours.

Evaluation of gene expression by quantitative real time PCR (qRT-PCR)

RNA was extracted using TRI reagent (Sigma, Roedermark ,Germany). To convert RNA into DNA, 20 µl reaction containing 1 µl random hexamer, 10 µl master mixes and 1000 ng of RNA samples were prepared. The qRT-PCR reaction was performed using 10 µl of 2X Master Mix (Biofact, Daejeon ,Korea). Temperature cycles included 15 minutes at 95 °C, 40 cycles at 95 °C for 15 seconds, 60 °C for 20 seconds, and 70 °C for 20 seconds using System LightCycler® 480 device (Roche, Basel, Switzerland). Table 1 shows the nucleotide sequence of the primers. $2^{-\Delta ct}$ was used to calculate the expression level of the targeted genes. The mean expression of Glyceraldehyde-3-Phosphate Dehydrogenase gene (GAPDH) was considered as an internal control to normalize gene expression level.

Evaluation of sclerostin and periostin secretion using ELISA

Human sclerostin and periostin ELISA Kit (Hangzhou EastBiopharm , Hangzhou, China) were used to evaluate the level of sclerostin and periostin in culture medium following the application of tensile and compressive forces. The experiment was performed according to the manufacturer's instructions. The absorbance was read at 450 nm wavelength using microplate reader (BioTek, Winooski, VT, Chittenden ,USA).

Evaluation of cell apoptosis using DAPI (6-diamidino-2-phenylindole) staining

DAPI staining was used to evaluate cell apoptosis after exposing the PDL cells to compressive stresses at 6, 24, 48, and 72 hours. For this aim, cells were fixed with paraformaldehyde 4% for 5 min followed by 3x wash with PBS. Then, cells were permeabilized with 0.2% Triton X100 in PBS for 5 min and incubated with DAPI 1:10000 (Santa Cruz Biotechnology, Dallas, Texas, United States). The cell's nuclei were examined under fluorescence microscope (Olympus,). The percentage of apoptotic cells with condensed and/or fragmented nuclei were determined by counting >100 cells per field from 5-6 different locations per slide.

Statistical analysis:

All experiments were repeated two times in triplicate. To examine the effect of two variables of time and type of applied forces on gene expression and protein production, two-way ANOVA was used. To examine the significance of time and force type interaction, one-way ANOVA was applied and t-test was used to compare the effect of compressive force with the tensile force applied at the aforementioned time intervals. P-value<0.05 was considered as the significance level.

Results

Morphological changes following the application of compressive forces

Following the application of compressive forces, changes were observed in the morphology and number of periodontal cells. The cell density decreased significantly and the cell morphology changed (Fig. 1).

POSTN, RUNX2 and SOST genes expressions following the application of compressive stress

There was a significant decrease in POSTN expression in a time-dependent behavior compared to the control group. The RUNX2 expression was significantly reduced at 24, 48, and 72 hours' time intervals compared to the control group. There was a significant increase in SOST expression after 6 hours and 24 hours compared to the control group. SOST expression was statistically different between the 6 hours and 48 hours groups as well as 72 hours and 6 hours groups (Fig. 2a).

POSTN, RUNX2, and SOST genes expressions after application of tensile stress

Following tensile forces, POSTN up-regulation was significant at 48 hours and 72 hours' time intervals compared to the control group. Also, RUNX2 expression was significantly increased at 48 hours and 72

hours' time intervals as compared to the control group. There was a significant decrease in SOST expression after 6 hours and 24 hours compared to the control group. Also, SOST expression was significantly different between 6 hours and 48 hours groups as well as 72 hours and 6 hours groups (Fig. 2b).

The expression of the apoptotic genes after application of compressive stress and tensile stress

The expression of PCNA decreased significantly after 6, 24, 48, and 72 hours, while the BCL2 gene expression increased after 24, 48, and 72 hours compared to the control group. Also, Caspase9 expression increased significantly after 24, 48, and 72 hours exposure to compressive force (Fig. 2c).

There were significant increase in the expression of PCNA after 72 hours tensile stress compared to the control group. BCL2 gene expression decreased significantly after 24, 48, and 72 hours. There was no difference in Caspase9 expression after 24, 48, and 72 hours compared to the control group (Fig. 2d).

Regression (correlation) of expression of different genes was also evaluated as shown in Figs 3a and 3b. There was a significant positive correlation between POSTN expression with RUNX2 and a negative correlation between RUNX2 and SOST expression as well as a significant correlation between SOST and POSTN following the application of tensile forces. Results also showed a positive significant correlation between POSTN and RUNX2 following the application of compressive forces.

Sclerostin and Periostin secretion level under tensile and compressive stresses

There was no significant change in sclerostin secretion level at each time points under the tensile stress. However, there was a significant increase in periostin production after 24 and 48 hours compared to the control group.

Sclerostin production was significantly increased in the compressive stress group after 24, 48, and 72 hours, while no significant change was observed in periostin level (Fig. 4).

Periodontal cell apoptosis following the application of compressive forces

The number of apoptosis in periodontal cells increased significantly 48 hours and 72 hours after the application of compressive forces as assessed by DAPI staining (Fig. 5).

Discussion

Sofar, different methods have been used to study the effects of mechanical forces on periodontal cells including applying periodic tensile and compressive forces, static tensile and compressive forces, hydrostatic pressure, centrifugation, vibration and liquid pressure. Among them, static compressive force provides the best simulation, mimics the compressive forces on the side subjected to compression.[15] In the present study, the in vitro model of compressive and tensile forces was used to evaluate the expression of periostin, and sclerostin, the key factors involved in bone remodeling, over the varied time

courses. For this aim, a total of 25 gr force (25 gr/cm2) was applied on silicone membrane at intervals of 6, 24, 48, and 72 hours to simulate orthodontic force on the compressive side. The tensile forces were applied with a frequency of 1 Hz and 10% elongation (strain) for 24, 48, and 72 hours.

Our results showed that there is a significant increase in sclerostin expression at gene and protein levels under compressive forces after 6 and 24 hours. While in the cells subjected to tensile forces, the expression of this gene and its protein were significantly reduced after 24 hours, indicating that the expression of sclerostin is dependent on the type of mechanical force.

In line with our results, Nishiyama et al., in an in-vivo study showed that compressive forces increase SOST gene expression and sclerostin protein level, while the opposite is true on the side under tension. They concluded that the contraction of collagen fibers in the areas under tension in periodontal ligament has a causative role in reducing sclerostin expression.[16] Considering the results of this study and the results of our in vitro study on cultured periodontal cells where no collagen fibers exist, it seems that a mechanism other than the contraction of collagen fibers is involved in the suppression of sclerostin expression on the tensile side. According to Ueda et al. in vitro study, light forces of 40g (16 g/cm^2) induce sclerostin expression in periodontal cells while applying forces of about 90 g reduce sclerostin level. The results were in contrast with their clinical study performed on rats model, indicating the sclerostin immune-reactivity on the weak pressure side. The difference was attributed to higher force applied in vivo compared to the force applied in laboratory evaluation.[1] Further clinical study by Premaraj et al. on rats model, showed that after applying a light force of 10 g, the expression of sclerostin gene increased within 24 hours in the areas under tension comparing to the areas under compression. After 48 hours, the expression of this gene increased in areas under pressure. However, the expression difference between compressive and tensile forces was not statistically significant, therefore, the expression of sclerostin was reported independent of the force type.[17] Although our results showed that the expression of sclerostin gene is affected by the type of force, factors such as hypoxia, the intensity and duration of force might affect the pattern of sclerostin expression.[18]

Studies have shown that periostin plays a key role in regulating the expression of proteinases secreted by periodontal cells such as cathepsin K, MMP1, and MMP2. Also it involves in the remodeling of collagen fibers upon mechanical loading.[19, 20] Our study showed that the expression of periostin gene, in addition to the type of force, depends on the time of exposure to mechanical force. The expression of periostin was significantly reduced within 24 hours after the application of compressive forces, while the application of tensile forces caused a significant increase in the periostin gene expression after 48 hours. In the study conducted by Panchamanon et al., compressive forces of 0.5, 1, 1.5 and 2 g/cm^2 on periodontal cells increased the expression of periostin, while values higher than 1 g/cm^2 showed suppressive effect, indicating that the response of periodontal cells depends on the magnitude of the applied force.[21]

RUNX2 is one of the most important transcription factors in the osteogenesis process, which plays a critical role in the initiation of osteogenic differentiation. This factor is an essential regulator of

osteoblast activity.[9] It has been well evident that SOST gene expression is controlled by RUNX2, and that knockdown of RUNX2 induces SOST expression indicating its inhibitory role on sclerostin expression.[22] Accordingly, our study showed that while sclerostin expression increased time-dependently in periodontal cells subjected to static pressure, the expression of RUNX2 is significantly reduced. The results were opposite under tensile force where the expression of RUNX2 showed increased, and the expression of sclerostin decreased significantly. Indeed, linear regression also confirmed significant correlation between these two genes in areas under tension. Consistent with our results, Tang's et al., investigated the effect of tensile forces on induction of osteogenic differentiation in periodontal cells and revealed that the application of periodic tensile forces increases the expression of transcriptional osteogenic factors RUNX2, and OSX after 6 hours.[23] Our results also showed a significant correlation between POSTN and RUNX2 expressions as well as between SOST and POSTN expressions. These data clearly suggested that light tensile forces induce osteogenic factors and inhibit negative regulators of bone formation.

We further evaluated the expression of genes involved in cell proliferation and apoptosis including PCNA, Caspase9, and BCL2. Proteins associated with the cell proliferation process such as PCNA are efficient markers in determining cell cycle progression and cell division. Indeed, a high expression of PCNA is the characteristic of the osteogenic cell that is a manifestation of DNA synthesis.[24]

Our findings showed that PCNA expression increased time-dependently after 72 hours exposure to tensile force. However, the application of compressive forces significantly reduced the expression of PCNA gene after 6 hours. The results of previous studies showed that applying tensile force to fibroblasts increases PCNA expression by 30–50% that was in accordance with in vivo studies and induction of PCNA + cells under tensile stresses in bone periosteal surfaces, which are the active areas for remodeling.[25] Also the expression of PCNA increases in the early stages of osteoblastogenesis and affects the functional characteristics of cortical bone, promote cell cycle from G1 to S phase.[26–28]

Previous studies have shown that the application of compressive forces involved with the induction of apoptosis and that the rate of apoptosis depends on the magnitude and duration of the force.[29] To examine the effect of compressive force on apoptosis, we further evaluated the expression of modulators of BCl-2-caspase-9 pathway. Our results showed that after 72 hours of applying compressive forces, the expression of BCL2 and caspase9 increased at the highest level while under tensile forces, the expression of caspase9 remained constant and the expression of BCL2 decreased to the lowest level after 72 hours. Therefore, unlike tensile force the application of compressive forces induced apoptosis in a time-dependent manner. Along with our result, Kassem H. et al. study on rat orthodontic tooth movement model showed that apoptotic osteocytes in both groups of light and heavy compressive forces increased significantly on pressure side compared to the tensile side. Although the distribution of apoptotic caspas3 + cells in the tension areas was unrelated to the force intensity.[30] According to the clinical study conducted by Leon et al., 8 months after the application of orthodontic force, the content of BCL2 protein in dental human pulp decreased in a time-dependent manner while the expression level of caspase9 increased. They reported that the orthodontic treatments reduced BCL2 expression time-

dependently, resulting in increased cell death. They showed that mechanical stress prevents the binding of BCL2 to apoptotic promoters such as BAX and BAK.[31]

Conclusion

The results of evaluation and analysis of data obtained from the present study are as follows:

Total SOST expression increased in areas undergoing compressive forces as compared to tensile forces in a time-independent manner and was influenced by the type of applied force.

The periostin production in areas undergoing compressive and tensile forces is different and this difference is affected by both variables of time and type of applied force. Applying compressive forces reduces genes expression involved in cell division and bone formation such as RUNX2, PCNA, POSTN, and increases expression of genes involved in apoptosis and bone resorption such as SOST, Caspase9. The application of tensile forces, in turn, will increase proliferative factors and factors promote bone formation such as RUNX2, PCNA, POSTN while reduced the SOST expression.

Declarations

Compliance with Ethical Standards:

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Acknowledgements

This study was part of M.S thesis supported by Tehran University of Medical Sciences

(grant No:70-02-98411975)

Funding

This study was part of M.S thesis supported by Tehran University of Medical Sciences

(grant No:70-02-98411975)

Consent to participate and for publication

This article is based on in-vitro study and has no any participate so it’s Not applicable.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by [mohammad sadegh ahmad akhoundi], [nastaran sharifi], [mahshid hodjat], [nooshin haghighipour] and [setareh kazemi veysari]. The first draft of the manuscript was written by [nastaran sharifi] and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Availability of data and materials

The datasets and/or analyzed during the current study are available from the corresponding author on reasonable request

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Tables

Table1: Primers Used for Quantitative Real-time Polymerase Chain Reaction for evaluation of expression of SOST,POSTN,RUNX2,CASP9,BCL2,PCNA

Genes	Nucleotide sequence (5’-3’)
beta actin -F	AAAACTGGAACGGTGAAGGT
beta actin -R	AACAACGCATCTCATATTTGGAA
SOST-F	AGACCAAAGACGTGTCCGAG
SOST-R	CCACTAGGTCGCCACCAC
POSTN-F	GCTGCCATCACATCGGACAT
POSTN-R	GGACACCTCGTGGAAGTTTCT
PCNA-F	TCTTCCCTTACGCAAGTCTCAG
PCNA-R	GGTTTACACCGCTGGAGCTA
BCL2-F	CTTCAGAGACAGCCAGGAG
BCL2-R	GTGTGGAGAGCGTCAACC
RUNX2-F	GGAGTGGACGAGGCAAGAGTT
RUNX2-R	GGTTCCCGAGGTCCATCTACT
CASPASE 9-F	AACCTTACCCCAGTGGTGCTC
CASPASE 9-R	ATCTGCATTTCCCCTCAAACTCTCA

Figures

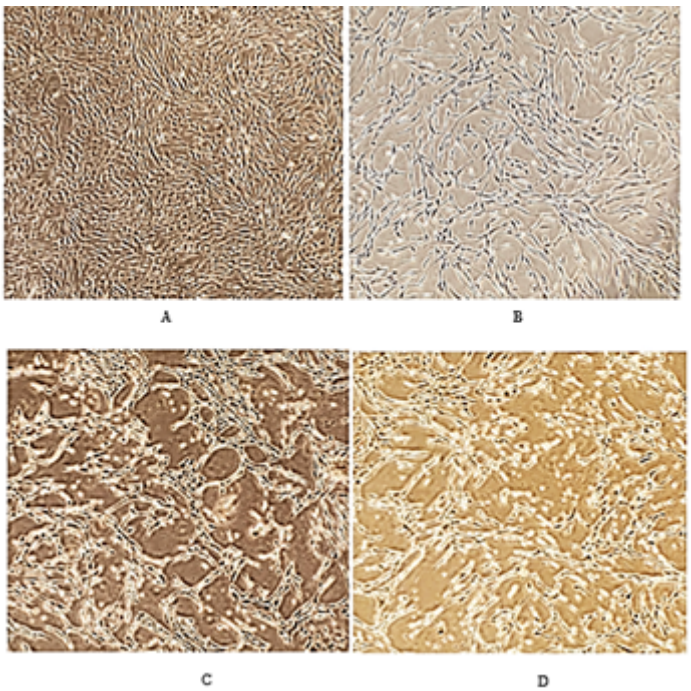

Figure 1

Morphological changes of periodontal cells under compressive forces after A:6hours,B:24 hours,C:48 hours,D:72 hours. periodontal cells undergoing compressive forces with a time-dependent decreased in density and morphology of these cells changed.

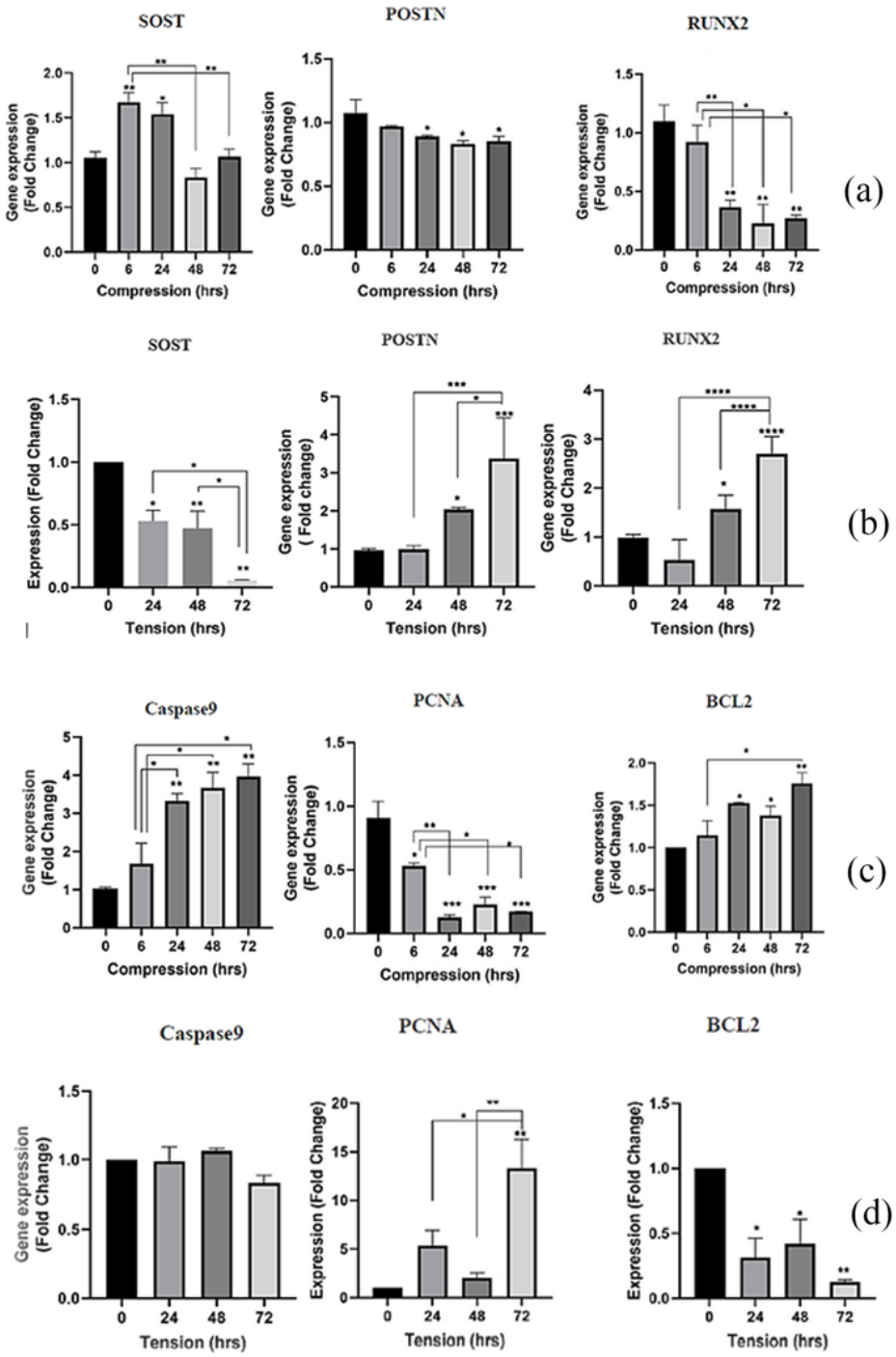

Figure 2

a)Resualts of RT-qPCR of SOST, POSTN, RUNX2 genes under compressive forces after 6, 24 ,48, 72 hours.expression of POSTN and RUNX2 after application of compressive forces significantly reduce and expression of SOST after 6 and 24 hourse significantly increased.b)Results of RT-qPCR of POSTN, RUNX2, SOST genes under tensile forces after 24,48,72hours.Expression of SOST after tensile forces significantly reduced, however expression of POSTN and RUNX2 increased.**c)**Resualts of RT-qPCR of PCNA, CASP9, BCL2 genes under compressive forces after 6,24,48,72hours.after application of compressive forces the expression of PCNA reduced and the expression of BCL2 and Caspase9 significantly increased.**d)** Results of RT-qPCR of PCNA, Caspase9, BCL2 genes under tensile forces after 24,48,72 hours.The expression of PCNA after tensile forces increased and expression of BCL2 in comparison of control group reduced and expression of CASP9 did not change.

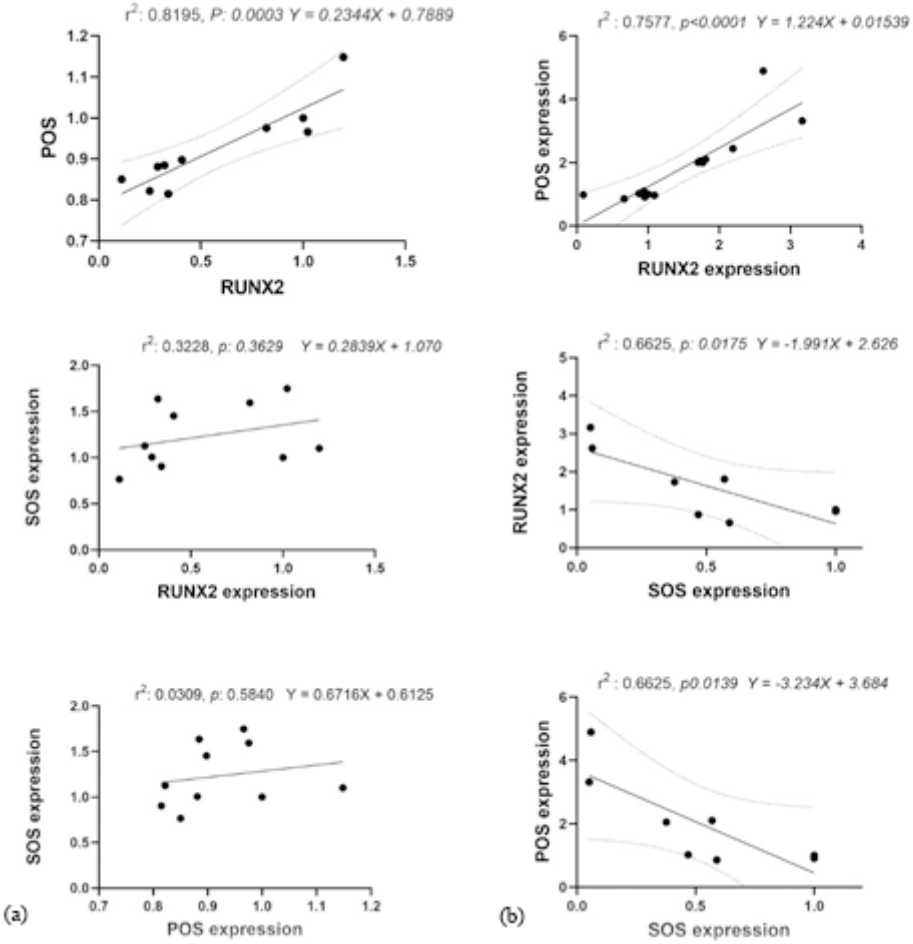

Figure 3

a)Regression analysis of genes expression under compressive forces. after application of compressive forces between RUNX2 and POSTN observed a positive correlation.b)Regression analysis of genes expression under tensile forces. after application of tensile forces between RUNX2 and POSTN observed a positive correlation and between RUNX2 and SOST,POSTN and SOST observed a negative correlation.

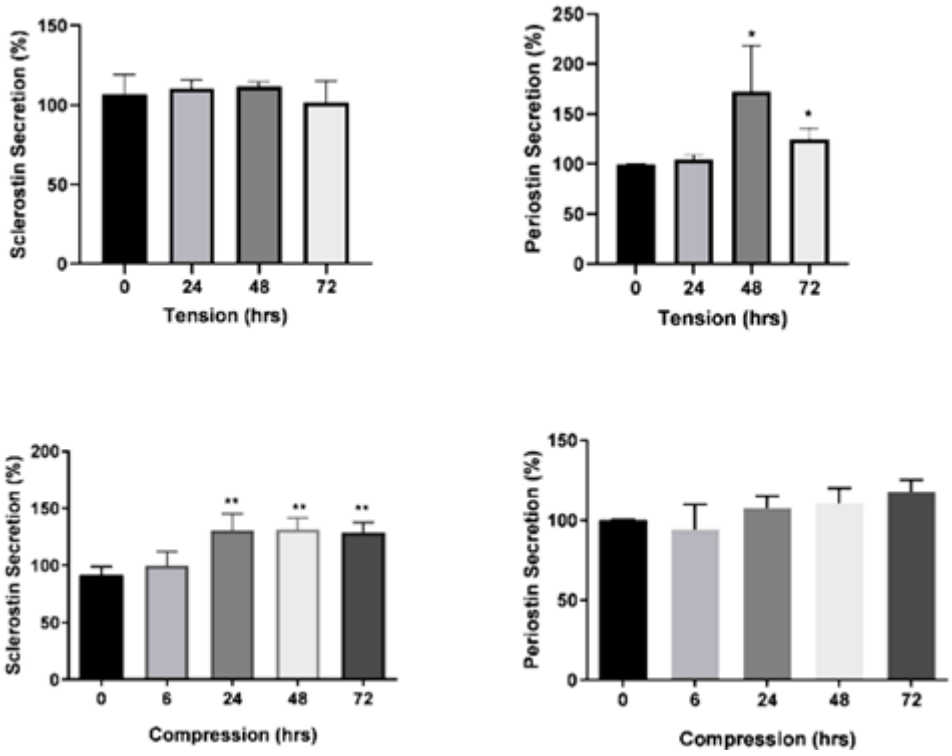

Figure 4

Secretin of periostin and sclerostin after compressive and tensile forces using ELISA. production of sclerostin after application of compressive forces significantly increased and production of periostin after48 and 72 hours application of tensile forces significantly increased .

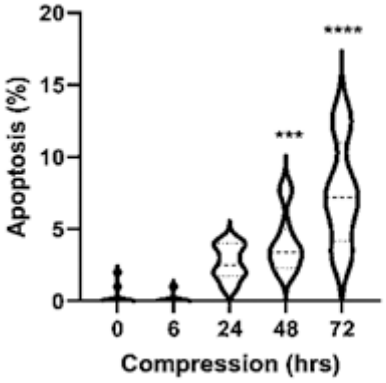

Figure 5

Percentage of apoptotic cells under compressive forces after DAPI staining. compressive forces resulted in a reduction of cell viability and with a time-dependent manner percentage of apoptosis was increased that confirms by chromatin condensation and increasing in fluorescent irradiation, at 48 and 72 hours after compression amount of apoptotic cells significantly increased.