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Evaluation of Mechanical and Chemical Stimulations on Osteocalcin and Runx2 Expression in Mesenchymal Stem Cells

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Abstract: The osseous tissue repair and regeneration have great importance in orthopedic and maxillofacial surgery. Tissue engineering makes it possible to cure different tissue abnormalities using autologous grafts. It is now obvious that mechanical loading has essential role in directing cells to differentiation.

In this study, the influence of cyclic uniaxial loading and its combination with chemical factors on expression of osteogenic markers was investigated. Rat bone marrow-derived stem cells were isolated and cultured. In one group cells were maintained in chemical induction medium. In another group cells were subjected to cyclic uniaxial strain with 3% amplitude and 0.3 Hz frequency for 24 hours and in the last group cells were affected by induction medium and physical stimulation. TaqMan Real time PCR and immunocytochemistry were done to evaluate gene expression variations. Moreover, a small incision was made to access the bone of the cranium and induced cells were seeded on collagen based scaffolds and finally the cell seeded scaffolds were implanted.

Results indicated that mechanical loading alone caused a phenomenal increase in Runx2 and osteocalcin expression. Remarkable increment in gene expression was gained when induction medium were added to mechanical stimulation. The order of chemical and mechanical stimulation caused different effects and results were much better when the cells were affected by mechanical strain at first. Histological analysis showed mechanical stimulation could promote bone ingrowth *in vivo*. These evidences demonstrated that combination of chemical factors with mechanical strain was much more effective for directing osteogenesis since these elements

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have synergistic effects.

Keywords: Cyclic stretch, Cranium, Mesenchymal stem cell, OCN expression, Osteogenesis.

1 Introduction

In the present century, along with the increase of lifespan, more skeletal diseases like bone cancer, rheumatoid, osteoporosis and trauma are observed [Salgado, Oliveira, Pedro, and Reis (2006); Meijer, De Bruijn, Koole, and Van Blitterswijk (2007)]. Unfortunately, it is predicted that the demand for bone grafts would be duplicated by 2020. Major reason is referred to changes in life style, including obesity and lack of physical activities [Amini, Laurencin, and Nukavarapu (2012)]. Hence, there is a tremendous gap in orthopedic and maxillofacial surgeries to return the functions of the skeletons and repair bone defects [Salgado, Oli- veira, Pedro, and Reis (2006); Meijer, De Bruijn, Koole, and Van Blitterswijk (2007)]. For this purpose, autografts became a gold standard. No risk of immunogenic reactions, disease transmission and infection are among the major advantages of autografts [Meijer, De Bruijn, Koole, and Van Blitterswijk (2007)]. Using stem cells in the form of autografts is a promising approach in treatment of many disorders due to their expansion potential and ability to differentiate to specific lineages [Heng, Cao, Stanton, Robson, and Olsen (2004)].

Stem cells are a kind of cells that remain in dormancy and undifferentiated state for a long time. They need specific signals to become activated and then start to differentiate [Shav and Einav (2010)]. It is confirmed that growth factors as chemical stimuli can promote stem cell differentiation [Salgado, Oli- veira, Pedro, and Reis (2006)]. Cells in tissues are continuously affected by different mechanical strains such as stretch, fluid flow and hydrostatic pressure [Shav and Einav (2010)]. These stimuli are pivotal for creating morphology in a single cell and even patterning of an organism [Shav and Einav (2010); Lee, Maul, Vorp, Rubin, and Marra (2007)]. Cells go through changes by altering their orientation, proliferation, gene expression and protein synthesis [Wang, Yang, and Li (2005)]. There is a relation between mechanical loading and cellular behavior. Type, amplitude and frequency of loading are the effective factors which should be considered in cell differentiation process [Safshekan, Shadpour, Shokrgozar, Haghighipour, and Alavi (2014); Haghighipour, Heidarian, Shokrgozar, and Amirizadeh (2012); Bayati, Sadeghi, Shokrgozar, Haghighipour, Azadmanesh, Amanzadeh, and Azari (2011); Norizadeh Abbariki, Shokrgozar, Haghighipour, Aghdami, Mahdian, Amanzadeh, and Jazayeri (2014)]. Regular exercise which leads to mechanical stimulation have essential contribution to bone formation and general healthiness of

bone [Bergmann, Body, Boonen, Boutsen, Devogelaer, Goemaere, Kaufman, Reginster, and Rozenberg (2010)] For example, in spinal injuries which necessitates long bed rest period, bone mass decrease is response not being exposed to mechanical forces [Delaine-Smith and Reilly (2012)]. Bones are under mechanical loads in a dynamic pattern *in vivo* and it is demonstrated that physiological loading and mechanical forces prevent bone loss with aging [Bhatt, Chang, Warren, Lin, Bastidas, Ghali, Thibboneir, Capla, McCarthy, and Gurtner (2007)]. Physiological strains play a key role in maintaining the hemostasis in musculoskeletal system [Bhatt, Chang, Warren, Lin, Bastidas, Ghali, Thibboneir, Capla, McCarthy, and Gurtner (2007)]. Mechanical strains such as compression, tension and shear are required for normal development of cells and exert their effects on regulation of the cell growth as well as its phenotype. Such activities recruit osteoprogenitor cells from bone marrow to differentiate into osteoblasts [Sittichokechaiwut, Edwards, Scutt, and Reilly (2010)]. It is well established that bone modeling, remodeling and even bone formation from progenitor cells sources are being enhanced by these mechanical forces [Koike, Shimokawa, Kanno, Ohya, and Soma (2005)].

The effect of mechanical stretch on gene expression pattern of mesenchymal stem cells has been studied before [Sittichokechaiwut, Edwards, Scutt, and Reilly (2010); Huang, Chen, Young, Jeng, and Chen (2009); Chen, Huang, Lee, Lee, Chen, and Young (2008); Wiesmann, Buhring, Mentrup, and Wiesmann (2006); Ignatius, Blessing, Liedert, Schmidt, Neidlinger-Wilke, Kaspar, Friemert, and Claes (2005)]. The results showed that low magnitude stretching causes osteogenic differentiation and expression of early genes [Chen, Huang, Lee, Lee, Chen, and Young (2008)]. It was elicited that cyclic tensile strain is a more effective approach compared to chemical supplements for promotion of osteogenesis [Huang, Chen, Young, Jeng, and Chen (2009)]. Additionally, physiological level of strain to human osteoblast cells could induce expression of early and late osteoblastic genes; Runt-related transcription factor 2 (*Runx2)* and osteocalcin [Bhatt, Chang, Warren, Lin, Bastidas, Ghali, Thibboneir, Capla, McCarthy, and Gurtner (2007)]. *Runx2* is considered to be a key gene which controls osteogenic differentiation. This gene was expressed in early phase of differentiation and regulated the transcriptional level of other bone-specific genes. *Runx2* plays its role as a molecular linker which connects mechanical stress to osteoblastic differentiation [Ignatius, Blessing, Liedert, Schmidt, Neidlinger-Wilke, Kaspar, Friemert, and Claes (2005)]. Collagen matrix development and maturation could be measured by expression of osteocalcin. Unlike *Runx2*, osteocalcin (*OCN*) was expressed at the late stage of osteoblastic differentiation before mineralization. The presence of OCN declares the fully maturation of osteoblasts [Wiesmann, Buhring, Mentrup, and Wiesmann (2006)].

The progressing need to bone grafts has developed a special branch in field of

tissue engineering called bone tissue engineering (BTE). BTE deals directly with bone structure, function and growth. Making practical cheap bone grafts is the main aim of BTE. Biocompatibility of scaffolds, osteogenic cells and morphogenic signals are among the most important issues must been regarded in BTE [Amini, Laurencin, and Nukavarapu (2012)]. Despite of vast researches in this area, there is still one main question unanswered. Which approach is the most convenient way for promotion of osteogenesis? It seems that this issue needs more studies. Due to established role of mechanical stimuli in osteogenic differentiation of mesenchymal stem cells, in this study, the effects of mechanical strains and chemical factors were investigated. In addition to that, by mixing these factors and changing the stimulation patterns, the effects of different combination pattern of mechanical forces and chemical factors were assessed. Quantified gene expression of Runx2 and OCN were measured by Real-Time PCR. The stimulated cells were cultured on scaffolds and implanted at the incision site on the cranium of the animal. Bone healing was assessed using histological experiments.

2 Materials and Methods

2.1 Rat bone marrow mesenchymal stem cells isolation

2.1.1 MSC isolation and culture

Animal experiments were done under the supervision of The Animal Rights Committee of Pasteur Institute. 9 male wistar rats (230–250 gr weight) were anesthetized using ketamine (50 mg/kg) and xylazine (5 mg/kg). Bone marrow aspirates were taken from iliac crest of the rats. Ficoll-paque technique was used to isolate stem cells from bone marrow. Culture medium was DMEM (Sigma, NY, USA) containing 15% FBS (Gibco, Heat-inactivated, NY, USA) and 1% penicillin/streptomycin (100 units/ml of penicillin and 100 ug/ml of streptomycin) (Sigma, NY, USA) and 1% L-glutamine (Gibco, NY, USA). The cells were subcultured and expanded to passage 3. The rat osteosarcoma cell line (UMR106) prepared by National Cell Bank of Iran (C586) was used as positive control and stem cells were used as negative control in all test groups of this study.

2.1.2 Bone marrow stem cells characterization

Flow cytometry was done to confirm the identity of the isolated cells. CD45, CD73 and CD90 surface antigens were selected. *CD73 and CD90* are positive in bone marrow stem cells and CD45 as a hematopoietic marker is negative for these cells. FITC-conjugated goat anti-mouse IgG antibodies were used for CD45, CD90 (FITC conjugated) and CD73 (PE conjugated antibody) (BD Biosciences, CA, USA). Secondary antibodies used in this study were Mouse IgG1 K Isotype Control

Purified (eBiosciences, CA, USA), Mouse IgG2a K Isotype Control FITC (eBiosciences, CA, USA) and Donkey Anti-Mouse IgG (H + L) PE (eBiosciences, CA, USA) for detection of CD45, CD90 and CD73, respectively. The related isotype control was utilized to determine the presence or absence of stains in comparison with the test group. Unstained cells were used for gating in all flow-cytometric analysis and at least 15000 events were counted. The achieved data were analyzed using version 7.6.4 of FlowJo analysis software [Chen, Huang, Lee, Lee, Chen, and Young (2008)].

2.1.3 Multipotency assays

Chemical compounds and growth factors were used to promote various differentiation pathways of MSCs and their ability to differentiate into adipogenic, chondrogenic and osteogenic pathways were examined. For adipogenic differentiation, the culture medium was replaced by medium supplemented with 1 μM dexamethasone, 500 μM isobutylmethylxanthine (IBMX), 60 μM indomethacin and 5 μg/ml insulin. Cells were cultured for 14 days and Oil-Red O staining was done to colorize lipoid vacuoles (all biochemical factors were purchased from Sigma, NY, USA).

For osteogenic differentiation, fresh medium were supplemented with 50 μM ascorbate-2 phosphate, 10 mM β-glycerophosphate and 0.1μM dexamethasone. Cells were cultured for 21 days. Alizarin Red S staining was used to visualize mineralized nodules in red (reagents were purchased from Sigma, NY, USA).

For chondrogenic differentiation, cells were pelleted and the medium was replaced by chondrogenic medium which contained 100 nM dexamethasone, 50 μM ascorbic acid-2 phosphate, 1 mM sodium pyruvate (Gibco, NY, USA), 10 ng/ml TGF β 1 (transforming growth factor-β 1; R&D Systems, MN, USA) and 1% ITS-Premix (BD Biosciences, CA, USA). Cells were cultured with this medium for 28 days and eventually, were fixed by 10% formalin (Sigma, NY, USA), sectionized and stained by Alician Blue (Sigma, NY, USA). Blue colored glycosaminoglycans were observable after Alician Blue Staining [Zuk, Zhu, Mizuno, Huang, Futrell, Katz, Benhaim, Lorenz, and Hedrick (2001)].

2.2 Application of mechanical stress

Cyclic uniaxial tensile stretch was applied to the cells by a device designed at Pasteur Institute of Iran [Haghighipour, Tafazzoli-Shadpour, Shokrgozar, Amini, Amanzadeh, and Khorasani (2007)]. Cells were subjected to strain on silicone membrane coated with collagen type I (0.5 mg/ml in 0.2% acetic acid) (Sigma, NY, USA). The MSCs at passage 3 were cultured on the precoated silicone membrane at a density of 5×10^5 and incubated an overnight for adhesion of the cells. Me-

Table 1: Designed primers, probes and their PCR products (Fw: forward, Re: reverse, Pr: probe)

Gene	Accession no.		Primer sequence	Amplicon size (bp)
RPL13	NM_031101.1	Fw	5′-TGAACACCAACCCGTCTCG-3′	109
		Re	5′-GCAGCCTGGCCTCTTTTG-3′	
		Pr	5′-CCCCTACCACTTCCGAGCCCCA-3′	
Runx2	NM_053470.2	Fw	5′-GCCAGGTTCAACGATCTGAGA-3′	86
		Re	5′-GGAGGATTTGTGAAGACCGTTATG-3′	
		Pr	5′-TGAAACTCTTGCCTCGTCCGCTCC-3′	
OCN	NM_013414.1	Fw	5′-GCAGACCTAGCAGACACCATGA-3′	79
		Re	5′-CCAGGTCAGAGAGGCAGAATG-3′	
		Pr	5′-TCTCTGCTCACTCTGCTGGCCCTG-3′	

chanical strain of 3% at 0.3 Hz was applied to the cells for 24 hours. Five different groups were designed as follows: 1) Cells were only induced by chemical factors (osteogenic medium) for 7 and 10 days, 2) Cells were stimulated merely by mechanical strain for 24 hours, 3) Cells were first subjected to mechanical strain for 24 hours and then cultured in osteogenic medium for 7 and 10 days, 4) Cells were first cultured in osteogenic medium for 7 and 10 days and subsequently, mechanical strain was applied for 24 hours. Control groups were maintained under identical culture conditions without mechanical stress.

2.3 Immunocytochemistry

To determine the differentiation along with osteogenic markers, cells were assessed for *Runx2* and *OCN* immunoreactivity, early and late phase markers of osteogenesis, respectively. Immediately after the end of each experiment, cells were rinsed twice with PBS and fixed in 4% paraformaldehyde (Sigma, NY, USA) for 20 min. Consequently, permeabilization step was carried out with 0.5% Triton X-100 (Merck, NJ, USA) for 10 min. Afterward, the cells were blocked by FBS before incubation with mouse monoclonal antibody to RUNX2 (1:100) and mouse monoclonal antibody to Osteocalcin (1:100) overnight at 4°C. In the next day, cells were rinsed three times with PBS and incubated for 2 hours with goat anti-mouse FITC conjugated secondary antibody (1:100) at room temperature and in a dark environment before being viewed by fluorescence microscopy (all antibodies were purchased from Abcam, Cambridge, UK) [Lee, Maul, Vorp, Rubin, and Marra (2007)].

2.4 Evaluation of gene expression

Total cellular RNA was extracted from MSCs using RNeasy plus Mini kit (Qiagen, MD, USA) according to manufacturers' protocol. Extracted RNA was stored in RNase free water at -80°C for further use. Concentration and quality of RNA was counted using nanodrop spectrophotometer. Only samples that were higher than 400 ng/μl were acceptable. Extracted RNAs were reverse transcribed into complementary DNA (cDNA) using QuantiTect Reverse Transcription kit (Qiagen, MD, USA). Quantitative real-time PCR analysis of mRNA levels was carried out using an ABI StepOne Real-Time PCR system (Applied Biosystems, CA, USA). Prepared *cDNAs were* checked by nanodrop spectrophotometer (A260/A280 $\geq$ 1.8) and gel electrophoresis. Primers and probes were designed using primer express software (version 3). The related sequences are shown in Table 1. *Runx2* and *OCN* genes were detected by Quantitative TaqMan RT-PCR assay. Each reaction was performed in a total volume of 20 μl containing 5 μl cDNA sample, 10 μl TaqMan Universal PCR Master Mix (Applied Biosystems, CA, USA), 10 pmol of each primer and 5′-Fam-/3′-Tamra-labelled probe. The cycle number was set to 40 and the annealing and extension temperatures were 60°C [Farrell, Byrne, Fischer, O'Brien, O'Connell, Prendergast, and Campbell (2007)]. In addition, the housekeeping gene, Ribosomal Protein Large subunit (*RPL-13*), was used as an internal control to calculate the difference in the Ct value of the target gene and control. *OCN* and *Runx2* values were normalized to *RPL-13* as endogenous reference. Relative gene expression was achieved using the following formula:

$$\begin{aligned}\Delta\Delta\text{CT} = &[\text{min CT Targets} - \text{min CT } \textit{RPL-13A}] \text{ Test samples} \\ &- [\text{min CT Targets} - \text{min CT } \textit{RPL-13A}] \text{ Stem cells}\end{aligned}$$

2.5 Surgical procedures

After the animal was anesthetized as described previously, a small incision was made on the left side of the cranium. To expose cranial bone, the soft tissues and the periosteum were removed and the collagen based scaffolds [Shokrgozar, Fattahi, Bonakdar, Kashani, Majidi, Haghighipour, Bayati, Sanati, and Saeedi (2012)] with diameter of $5\times5\times1$ mm^3 were implanted after predrilling with dental drill. Three different groups were designed as follows: group 1: bone sockets without any scaffolds, group 2: defects filled by undifferentiated MSCs seeded scaffolds and group 3: defects filled by mechanically and chemically differentiated MSCs seeded scaffolds. The incisions were closed using a vicryl 3-0 suture. It must be mentioned that the isolated cells from each animal's body were grafted to itself. After 10 weeks of implantation, the scaffolds were retrieved, fixed in 4% paraformaldehyde for 12h and bones were decalcified in 10% EDTA for two weeks and then embedded

in paraffin. The tissue blocks were sectioned at 5 μm thickness and stained with hematoxylin and eosin (H&E) for general assessment of bone healing.

2.6 Statistical analysis

Each experimental test was run in triplicate. Mean and standard deviations (SD) were calculated in collecting the results. They were achieved using SPSS software version 16.0. One way analysis of variance (ANOVA) was performed to analyze the mean values statistically. Values were considered significant at $p < 0.05$.

3 Results

3.1 Characterization of MSCs

3.1.1 Flow cytometric characterization of MSCs

Flow cytometry was done to characterize the cultured cells. It should be noted that all cells used in the characterization experiments were in P3. Cells expressed CD73 (69.8%) and CD90 (97.6%) on their surface. In contrast, the expression of CD45, as hematopoietic stem cells marker, was very rare (0.5%) (Data not shown).

3.1.2 Differentiation potential assays

The potential of cells to differentiate into osteogenic, adipogenic and chondrogenic lineages was also evaluated. Alizarin red s staining was done on day 21 to stain calcified nodules for osteogenic differentiation (Fig. 1a). Oil red o staining on day 14 showed lipidic vacuoles in adipogenic differentiation (Fig. 1b) and Alician blue

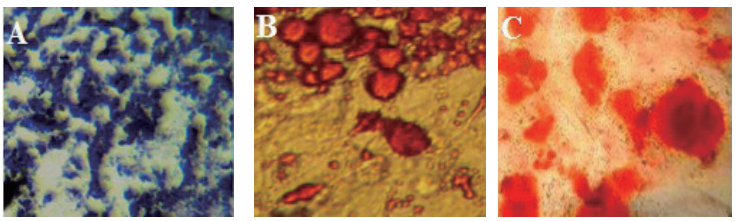

Figure 1: Multipotency assays. Differentiation potential assays of isolated BMSCs at 3th passage. (a) A section of pelleted MSCs after differentiation by chondrogenic medium, blue color indicates glycosaminoglycan after staining with Alician blue (Optical microscope, 250×). (b) Staining with oil red O stains neutral lipids and showed lipidic vacuoles (inverted microscope, 400×). (c) Alizarin red S staining was used to show calcium deposition by dark red spots (400×).

staining was done on day 21 to reveal sulfated glycosaminoglycan and chondorogenesis (Fig. 1c). Due to the potency of differentiation into these 3 lineages, the stemness of the cells was verified.

3.2 Immunocytochemistry

Immunocytochemistry conclusively demonstrated small amount of *Runx2* protein in stem cells (Fig. 2a) but no osteocalcin protein was observable (Fig. 2c). Cyclic mechanical strain promoted *Runx2* expression (Fig. 2b) and stimulated osteocalcin expression (Fig. 2d). These data indicate expression of osteoblastic genes and osteogenic differentiation of MSCs.

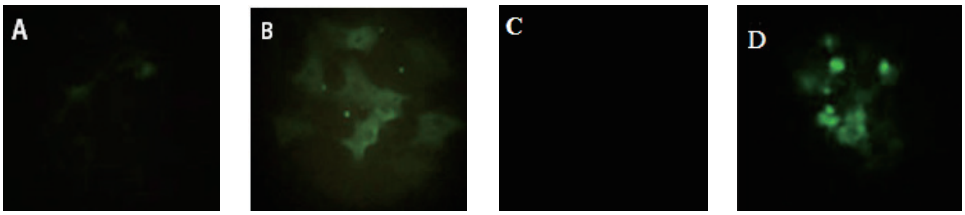

Figure 2: Localization of osteoblast specific proteins using immunocytochemistry. *Runx2* and *OCN* were characterized by immunocytochemistry in unstretched mesenchymal stem cells (a, c). The cells were seeded on silicone membrane and a regimen with 3% strain and 0.3 Hz frequency was applied for 24 hours. Immunocytochemistry revealed existence of *Runx2* (b) and *OCN* (d). The fluorescence color was visualized using Carl Zeiss fluorescent microscope, 630×.

3.3 Characterization of osteogenic gene expression

The levels of *OCN* and *Runx2* mRNAs were assessed using TaqMan Real-Time PCR. In the cells cultured in medium supplemented with osteoinductive factor, gradual increase in expression of *Runx2* and *OCN* were seen which reached its maximum at day 10 (Fig. 3).

After 24 hours of continuous cyclic mechanical loading (3% stretch amplitude and 0.3 Hz frequency), the expression level of *Runx2* was lower than UMR106 and higher than MSCs (Fig. 4a). On the other hand, the expression levels of *OCN* were higher than UMR106 (1.6 fold) (Fig. 4b). It is probable that the expression of *Runx2* reached its maximum before the time of RNA extraction (after 24 hours).

The expression level of genes was higher in the mechanically stretched cells which had passed 7 days of chemical induction after mechanical stress (Fig. 4).

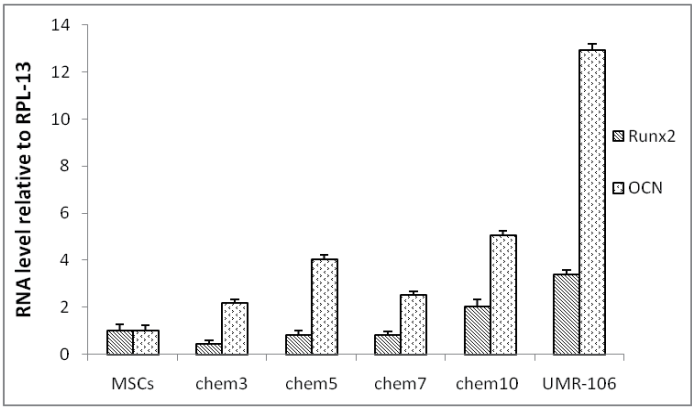

Figure 3: Evaluation of gene expression in chemical induction group. mRNA level of *Runx2* (a) and *OCN* (b) is shown in chemically stimulated cells (after 3, 5, 7 and 10 days) UMR106 (positive control). *Runx2* and *OCN* level reach their maximum amount after 10 days of adding Chemical induction medium. *Chem: After days of chemical induction. Calculated P Value is < 0.05.

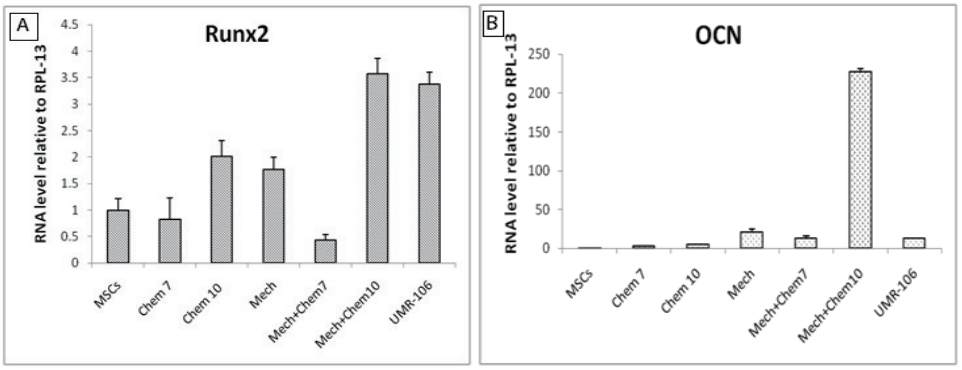

Figure 4: Evaluation of gene expression in chemical induction, mechanical loading and their combination. mRNA level of *Runx2* (a) and *OCN* (b) is shown in chemically stimulated cells (after 7 and 10 days), mechanically stretched cells (after 24 hours), Mech + Chem. stimulated (after 24 hours of tensile strain + 7 and 10 days chemical induction), UMR106 (positive control). *Runx2* had its maximum amount in the Mech + Chem10 group. *OCN* level had their maximum amount in the Mech + chem10 group. *Chem7: After 7 days of chemical induction. Calculated *P* Value is < 0.05.

Table 2: The effect of arrangement of loading and chemical induction on expression level of Runx2 and OCN

Sample	***Runx2***	***OCN***
24 h loading + chemical induction (10 days)	3.575	227.286
chemical induction (10 days) + 24 h loading	0.34	5.5

The expression level of *Runx2* in mechanical/chemical group was equivalent to that of UMR106 in the cells which passed 10 days of chemical induction after mentioned mechanical loading. At this time, slightly higher expression level was observed in chemical induction in comparison to mechanical group. In mechanical/chemical group, a significant increase in *OCN* gene expression was observed compared to UMR106 (about 170 fold). The strained cells showed higher levels of gene expression compared to chemically stimulated group (Fig. 4).

The survey results revealed that the expression of differentiation markers, particularly the late stage gene *OCN* was higher in the samples which were exposed to the uniaxial strain at first and then were stimulated with induction medium (Table 2). In the first group, mechanical loading was first applied to the cells and then they were placed in a chemical induction medium. In the second group, the situation was reversed: cells were first subjected to the induction medium and in the next step, they were mechanically stretched.

3.4 In vivo studies

The potential of bone tissue in-growth of the differentiated MSCs under the applied mechanical loading was assessed by histological analysis. Generally, no noticeable inflammatory cells such as macrophages, lymphocytes and giant cells were observed after 10 weeks of implantation in all groups. Areas with osteoid could be identified next to preexisting bones for all test groups (Fig. 5). This is due to the activity of abundant osteoblast cells that produced osteoid on the surface of the new bone. In addition, there were no signs of implanted scaffolds due to degradation. In general, defects in all groups were filled with new bone after 10 weeks.

4 Discussion

The present research was designed to evaluate the effects of cyclic tensile stretch and chemical induction separately and concurrently on differentiation of MSCs into osteoblast and restoration of injured bone tissue. Also in combination sector, the effect of the application priority of mechanical forces or chemical factors was assessed. The efficacy of these three inductions was studied by applying im-

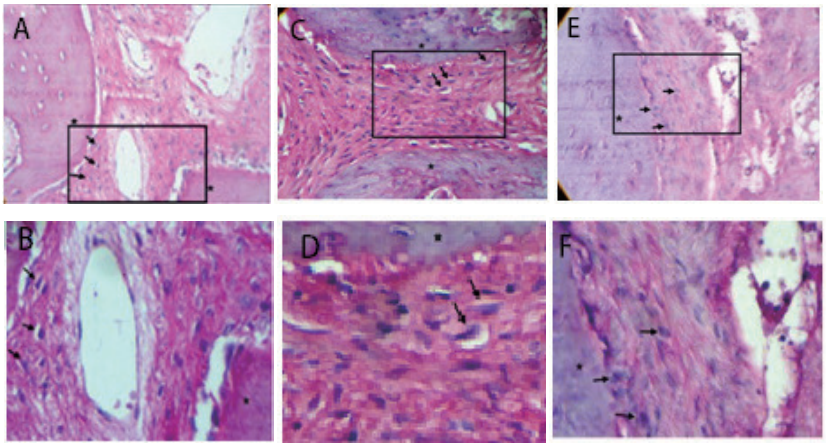

Figure 5: Histological analysis. The *in vivo* bone formation was assessed by hematoxylin and eosin. Bone sockets without any scaffold (negative control) (a, b), defects filled by undifferentiated MSCs seeded scaffolds (c, d), defects filled by mechanical differentiated MSCs seeded scaffolds (e, f).

munocytochemistry, TaqMan Real-Time PCR and histological methods. Two gene markers, *Runx2* and *OCN* were selected as osteogenic indicators and RPL-13 was utilized as housekeeping gene to normalize the results. The healing potency of the cells that were seeded on scaffold was assessed by H&E staining and histological analysis.

The works of some previous researchers indicate that strains with small amplitude prevent proliferation of bone cells and excite their differentiation. Moreover, the physiological loads that bones bear in the body are very low [Mullender, El Haj, Yang, Van Duin, Burger, and Klein-Nulend (2004)]. Another report demonstrated that only cyclic strain can induce new bone formation. This report mentioned the decisive role of amplitude and duration of applied strain on differentiation. The effects of high amplitude and short loading duration was similar to that of low amplitude and longtime loading duration [Duncan and Turner (1995)]. According to reports, the adequate mechanical stretch required to stimulate osteoblasts *in vitro* is 10–100 times higher than such strains *in vivo* [Turner and Pavalko (1998)] and the resultant physiological loads on bone is between 0.2–0.3% of strain [Mullender, El Haj, Yang, Van Duin, Burger, and Klein-Nulend (2004)]. Therefore, 3% stretch was applied in this study. While it takes 24 to 48 hours for new osteoblasts to emerge after initiation of mechanical loading, bone formation is observable within 96 hours of initiation of loading [Roosa, Turner, and Liu (2012)]. Thus, the duration time of mechanical loading in present study was set to 24 hours.

The results of Real time PCR showed expression of *Runx2* and *OCN* in mechanically stimulated groups. It was evident that the expression of *Runx2* was increased in mechanical loading group in comparison to the group in which cells were subjected to osteogenic induction medium for 7 days. This increase was also observable in the expression of *OCN* in mechanical loading group compared to chemically induced cells for both 7 and 10 days. Therefore, given that the duration of mechanical loading required for expression of marker genes is less compared to that of exposure to osteogenic medium and on the other hand, the expression level of marker genes is higher in mechanical loading groups, it can be inferred that mechanical loading is a more efficient approach to stimulate the osteogenic differentiation of bone marrow derived mesenchymal stem cells compared to chemical induction [Huang, Chen, Young, Jeng, and Chen (2009); Wiesmann, Buhring, Mentrup, and Wiesmann (2006); Qi, Zou, Han, Zhou, and Hu (2009)].

The expression of *Runx2* and *OCN* reached their maximum level after 24 hours of cyclic mechanical stimulation followed by 10 days of chemical induction (mechanical + 10 days chemical). This result is in agreement with previous studies in the literature, although there are some differences such as duration of stimulation, number of cycles and the time of exposure to osteogenic medium. By simultaneous use of mechanical stimulation and chemical induction, the expression of *OCN* was observed after 14 days [Wiesmann, Buhring, Mentrup, and Wiesmann (2006)].

Mechanical stretching is the most pivotal signal that involves in bone remodeling and osteoblast functions via mechanotransduction. Runx2 operates regulatory role via a complicated signaling pathway. In summary, integrins probably sense mechanical strain and ignite the Ras-extracellular signal-regulated kinase 1/2 (ERK1/2) mitogen-activated protein kinase (MAPK) signaling pathway. In this way Runx2 is phosphorylated and thus capable to do transcriptional activity. In other words phosphorylated Runx2 is apt to binding to DNA motifs in the position of osteoblast-specific cis-acting element (OSE2). OSE2 is located in the promoter of the major osteoblast-related genes. The OSE2 position helps Runx2 to modulate the expression profile of the mentioned genes. According to the defined signaling, mechanical stress has a critical role in osteoblastic differentiation via Runx2 function [Ziros, Basdra, and Papavassiliou (2008)]. However one of the pathways trigger mechanotransduction in the cells is integrin-cytoskeleton complex and this pathway is a direct linker between transmembrane integrins that attached to cell membrane and actin cytoskeleton in cytoplasm and the transcription machinery located in the nuclear [Turner and Pavalko (1998)].

Another mechenotransducer is G protein. This protein has interaction with stretch-activated cation channels and other pathways and molecules such as inositol triphosphate, cyclo-oxygenase (COX) and nitric oxide synthase (NOS). Mechani-

cal stresses cause calcium (Ca^{2+}) to release from intracellular reservoir or come through membrane channels which leads to increasing of the amount of Ca^{2+} in cytoplasm. This calcium accession excites second messengers like nitric oxide and prostaglandins, these molecules act in two forms. In the first form these messengers stay within the cell cytosol and modulate gene expression in the nuclear. In the other form the messengers move out of the cell and ignite autocrine and paracrine signaling [Turner and Pavalko (1998)].

In the next step, MSCs were exposed to the combination of the mechanical forces and chemical induction and also the effect of priority (which stimulus was applied first) was assessed that has not been done in previous studies. In general, the obtained data revealed that it is more effective to first apply the mechanical loading to the cells and then add the induction medium. In this regard, after 24 hours of loading and then 10 days of exposure to induction medium, expression of Runx2 and *OCN* were increased 9 and 40 times, respectively. It was proved that the mechanical loading had a greater ability than chemical factors to augment osteogenic differentiation.

In the present work, the *in vivo* results provide evidence that scaffold seeded with differentiated osteoblast cells support *in vivo* healing of the defect. Histological sections stained with H&E of different implant types exhibited new bone tissue formation throughout the structure of the scaffolds after 10 weeks. In all groups, many new osteoblast cells produced osteoid on the surface of the preexisted bone with no noticeable fibrous tissue formation and inflammation response. According to the obtained results, mechanical loading could differentiate MSCs to active osteoblast cells with the ability to participate in bone regeneration.

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