Research Article

Effect of Platelet-Rich Plasma on Differentiation of Osteoblasts and Osteoclasts in the Presence of Three-Dimensional Scaffold

Hadi Mokhtari[1], Azadeh Montaseri[2], Mohammad Ali Mojaddadi[1*], Hamidreza Mokhtari Zonouzi[1], Nahid Karimiyan[2], Saba Arami[3]

[1]Department of Endodontics, Faculty of Dentistry, Tabriz University of Medical Sciences, Tabriz, Iran.
[2]Stem Cell Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.
[3]Department of Aesthetic and Operative, Faculty of Dentistry, Tabriz University of Medical Sciences, Tabriz, Iran.

A r t i c l e   I n f o

A B S T R A C T

Background: Osteoblasts' activity is prerequisite for prevention from and treatment of apical periodontitis and a relatively high proportion of endodontically treated teeth will require retrograde treatment in future. Therefore, the aim of the present study was to evaluate the effect of platelet-rich plasma (PRP) on differentiation of stem cells into osteoblasts and osteoclasts.
Methods: Mesenchymal stem cells were isolated from human fetal umbilical cord and cultured on two polycaprolacton/hydroxyapatite (PCL/HA) polymer scaffolds. In addition to differentiation agents, 10% PRP was added to PRP containing subgroups. After 10 days, osteoblast differentiation was assessed evaluating the osteocalcin and osterix gene levels where, in the osteoclast differentiation group the expression of tartarate-resistant acid phosphatase (TRAP) gene was evaluated.
Results: Expression of TRAP gene did not reveal any significant differences between the study and control groups. There was a significant difference in osterix expression between the control and the PRP-treated groups ($p < 0.01$) as well as osteocalcin gene ($p < 0.05$).
Conclusion: The results showed that PRP increased the osteoblastic differentiation, while it does not cause any significant increase in osteoclastic differentiation.

Introduction

Generally, the success rates of orthograde treatments in endodontics in teeth with lesion and in vital teeth are 72% and 83%, respectively.[1] In addition, the success rates of non-surgical pretreatments and endodontic surgeries without the use of guided bone regeneration (GBR) techniques have been reported to be 78.2% and 63.4%, respectively.[2] In other words, a relatively high percentage of teeth will need retrograde (surgical) treatment in future. Recently, tissue engineering is introduced as a new branch of science for designing and synthesizing different tissues and organs consisting of cells, scaffolds, growth factors and bioreactors.[3]

Long-term survival of differentiated cells in the 3D environment is the principal advantage in tissue engineering, which might be the key to the use of these scaffolds in animal studies on medications, gene therapy, cancer biology and, regenerative treatments.[3]

There are genetic controllers of osteoblast and osteoclast differentiations which are reported before. Osteoblast differentiation can be controlled in transcriptional level by core-binding factor alpha-1 (Cbfa-α), while secreted molecular factors are bone morphogenetic proteins (BMPs), Indian hedgehog (Ihh). Osteoclast differentiation can be studied in transcriptional level using osteopetrosis (op/op), PU.1, c-fos, nuclear factor kappa B (NF-kB), c-Src and, Microphthalmia. Secreted molecular factors involved in osteoclast differentiation are macrophage colony-stimulating factor, osteoprotegerin and, receptor activator of NF-kB and its ligand.[4]

Usage of platelet-rich plasma (PRP) is an innovation in regenerative techniques. Growth factors derived from platelets initiate connective tissue repair and bone regeneration which result in an increase in neoangiogenesis and simulation of wound repair processes.[5,6]

PRP refers to an increased concentration of platelets that have been packed in a small volume of plasma. It is derived from centrifugation of fresh blood. Platelets contain α- granules, the contents of which are released during the few first minutes of activation and contain growth factors such as platelet-derived growth factor (PDGF), transforming growth factor (TGF), insulin-like growth factor (IGF), prostaglandin E2 (PGE2) and vascular endothelial growth factor (VEGF).[7,8]

In 1997 Whitman was the first researcher to use PRP in oral surgeries.[9] Marx introduced the use of PRP in the regeneration of the alveolar bone[8] and reported more favorable bone maturation and its radiographic density with the simultaneous use of autologous graft and PRP

compared to the use of autologous graft alone. The risk of infection transmission and the immune response rate are low using PRP. The antimicrobial activity of PRP is another property that has been reported against some microorganisms responsible for oral cavity infections.[10-13] Mesenchymal stem cells (MSC) were isolated for the first time by Friedenstein from bone marrow. The MSCs have high potential for spontaneous proliferation and differentiation into cells that form cartilage, bone, adipose tissue, skeletal muscles and connective tissue stroma. The umbilical cord is available during birth without any injuries to the newborn and has superb clinical potential for use as an autologous tissue. These MSCs can differentiate into all cell types.[14]

Considering the importance of complete simulation of the oral cavity conditions in three-dimensional culture media, the aim of the present study was to evaluate the effect of PRP on differentiation of human umbilical cord derived mesenchymal stem cells into osteoblasts and osteoclasts.

Materials and Methods

The polycaprolacton/hydroxyapatite (PCL/HA) polymer scaffold was purchased from Stem Cell Technology Research Center (Tehran, Iran). In the present study, the cells were seeded on polycaprolacton/hydroxyapatite (PCL/HA) polymer scaffolds that allowed free penetration of stem cells into the scaffold. The scaffolds were cut with the use of a sterilized biopsy punch into segments with a diameter of 6 mm and their final sterilization was carried out with UV light.

In order to isolate stem cells from umbilical cord of healthy human fetus, the umbilical cord was washed in ethanol 70% solution for 30 seconds and kept in fresh normal saline liquid. The blood vessels and amniotic layer of the segmented umbilical cord was separated after washing in PBS buffer and the wharton jelly parts were placed gently in PBS and washed in. the obtained cells which were the mesenchymal stem cells were placed in cell culture T flask containing Dulbecco's Modified Eagle's Medium (DMEM) (Biosera, Hameenlinna, Finland) containing 10% fetal bovine serum (FBS) and 100 µg/mL streptomycin and 100 units/mL penicillin G. the cells were incubated at 37^{0C} in 95% humidity and 5% CO_2 condition. The media was freshened after 3-4 days until the cells reach to 70-80% confluency. The cells were passaged through enzymatic methods and the process was repeated until 4th subculture. Then, the supernatant was collected and frozen as conditioning medium for further assays. The cells were ready to be cultured on scaffolds.

One million stem cells isolated from the Wharton jelly of the human umbilical cord were seeded on the polymer scaffolds. The cells used in the present study were divided into 6 groups: the control group; the group for differentiation into osteoblasts in which only the culture media was used in association with agents that help stem cells differentiate into osteoblasts; the group for differentiation into osteoclasts; the PRP-treated group; the PRP-treated group for differentiation into osteoblasts; and PRP-treated group for differentiation into osteoclasts. In order to induce differentiation of stem cells into osteoblasts, DMEM medium containing 10^{-7} mol of dexamethasone (Sigma-Aldrich, Missouri, US), 10 mmol of β-glycerophosphate (Sigma-Aldrich, Missouri, US) and 50 mmol of ascorbic acid (Sigma-Aldrich, Missouri, US) was used.

In the osteoclast differentiation group, the stem cells derived from the Wharton jelly were transferred to the scaffolds and induced to differentiate into osteoclasts with the use of receptor activator of nuclear factor Kappa-B ligand (RANKL) (Peprotech, London, UK) (100ng/mL) and colony-stimulating factor (CSL) (Peprotech, London, UK) (20 ng/mL). The cells in the PRP-treated groups were subjected to 10% PRP in addition to the agents used to induce differentiation. The cells were stored for 14 days and at the end of the study were prepared for evaluation of genes.

The expression of specific osteoblast and osteoclast genes was evaluated in order to determine the differentiation of stem cells isolated from Wharton jelly into osteoblasts and osteoclasts. For osteoblasts, osteocalcin and osterix genes were evaluated with real-time PCR. The expression of TRAP was evaluated to investigate differentiation of stem cells into osteoclasts.

Total cellular RNA was extracted using YTA mini kit (Cat.NO: YT9065, Taiwan) according to the manufacture's protocol. Briefly, after centrifugation and supernatant removal, RB Buffer was added to the cell pellet to lyse the cells. Then sample mixture was transferred to the collection tube containing filter column and centrifuged at 14000 rpm/2 min. In the next step, samples were mixed with 70% ethanol. RB mini column was washed at first with wash buffer 1 and subsequently with wash buffer 2. Finally, RNase–free ddH$_2$O was added to the samples and centrifugation at 14000 rpm/2 min was performed to elute RNA. Approximately 1000ng/1 mL of total RNA was used as template for cDNA synthesis using reverse transcription kit (Takara, RR037I, Japan).

Table 1. Primer sequences used in Real Time RT-PCR and related annealing temperatures.

Gene	Primer	Annealing temperature (°C)
OC-F	CTGACCTCACAGATGCCAA	54
OC-R	GGTCTGATAGTCTGTCACAA	54
OSX-F	CCTCTCGACCCGACTGCAGATC	66
OSX-R	AGCTGCAAGCTCTCTGTAACCATG	66
TRAP-F	GCAGACCAGGGAAACTGAAGCA	65
TRAP-R	CGTTGATGTCGCACAGAGGGAT	65
GAPDH-F	TGAGGTGACCGCATCTTCTTG	62
GAPDH-R	TGGTAACCAGGCGTCCGATA	62

The Real time PCR reactions were performed using a (Corbett, 010755, Australia) system with a SYBR Green master mix (Takara, RR820L, Japan) under the condition of 15s at 95°C and different time interval and annealing temperature for each gene as can be found in Table 1. The primer sequences used for this investigation are also listed in Table 1. The gene expression levels were calculated using Pfaffl formula and GAPDH was used as internal control. All experiments were done in triplicate.

All data are reported as means ± SD. Analyzing with Graphpad software, statistical difference between two groups was determined by one-way ANOVA, two-way ANOVA and followed by post hoc tukey test. P-value less than 0.05 was set as significant.

Results

The expression of TRAP, osterix and osteocalcin genes was analyzed with real-time PCR in 6 study groups.

As shown in Figure 1, expression of TRAP gene was not significantly different between the PRP-treated and control groups ($P \geq 0.05$). The difference between the osteoclastogenesis marker group and the group with osteoclastogenesis marker +PRP was significant ($P < 0.05$).
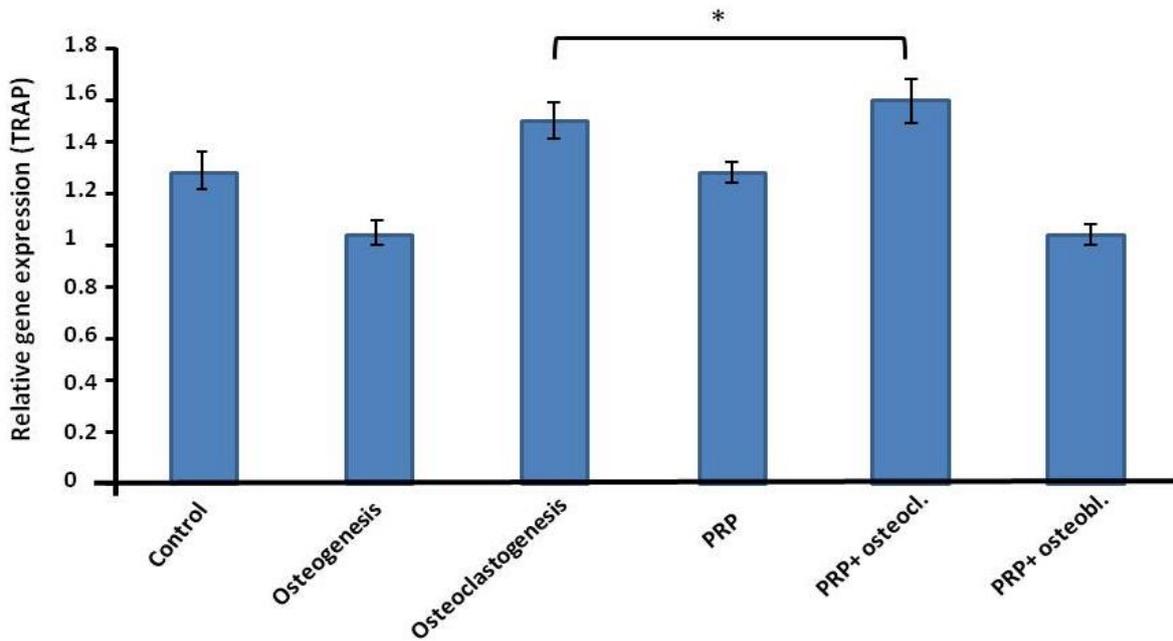

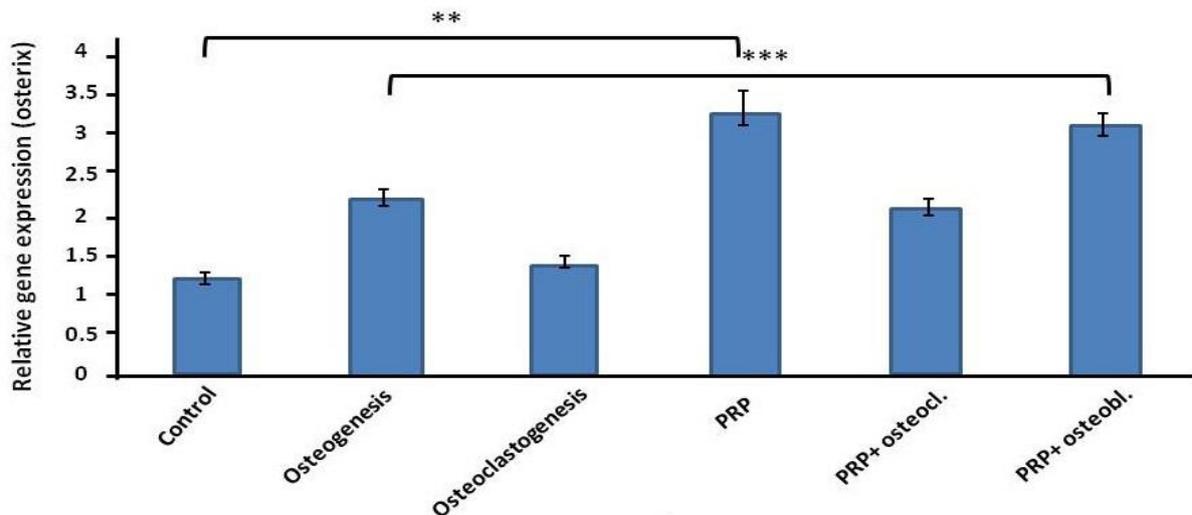**Figure 1.** Comparison of TRAP gene expression ratio among different treated groups. * represents p < 0.05.

Figure 2. Comparison of Osterix gene expression ratio among different treated groups. ** and *** represent p < 0.001 and p < 0.0001, respectively.

As shown in Figure 2, comparison of the expression of osterix gene between the PRP-treated and control groups showed a significant difference between them ($p < 0.01$). The difference between the groups with osteoblastogenesis marker alone and the group with the marker above plus PRP was significant ($p < 0.001$).

Evaluation of the expression of osteocalcin, as shown in Figure 3, revealed a significant difference between the PRP-treated group and the control group ($p < 0.05$); however, comparison of the two osteoblastogenesis

marker groups with and without PRP revealed no significant differences ($p > 0.05$).

Comparison of the effect of PRP on the expression of the three genes mentioned above with the use of post hoc Tukey tests (Figure 4) showed significant differences between the expression of osterix and osteocalcin genes ($p < 0.05$ and $p < 0.001$, respectively). Based on Figure 4, intervention meant use of PRP and values 1 to 3 indicate genes TRAP, osterix and osteocalcin, respectively.
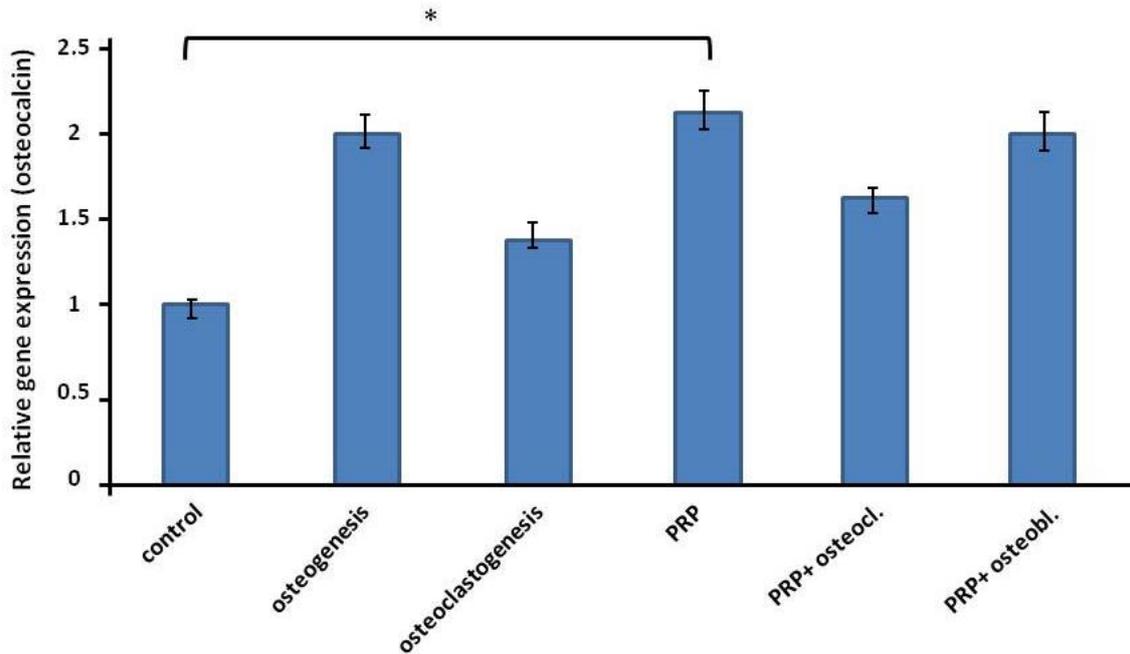

Figure 3. Comparison of Osteocalcin gene expression ratio among different treated groups. * represents $p < 0.05$.
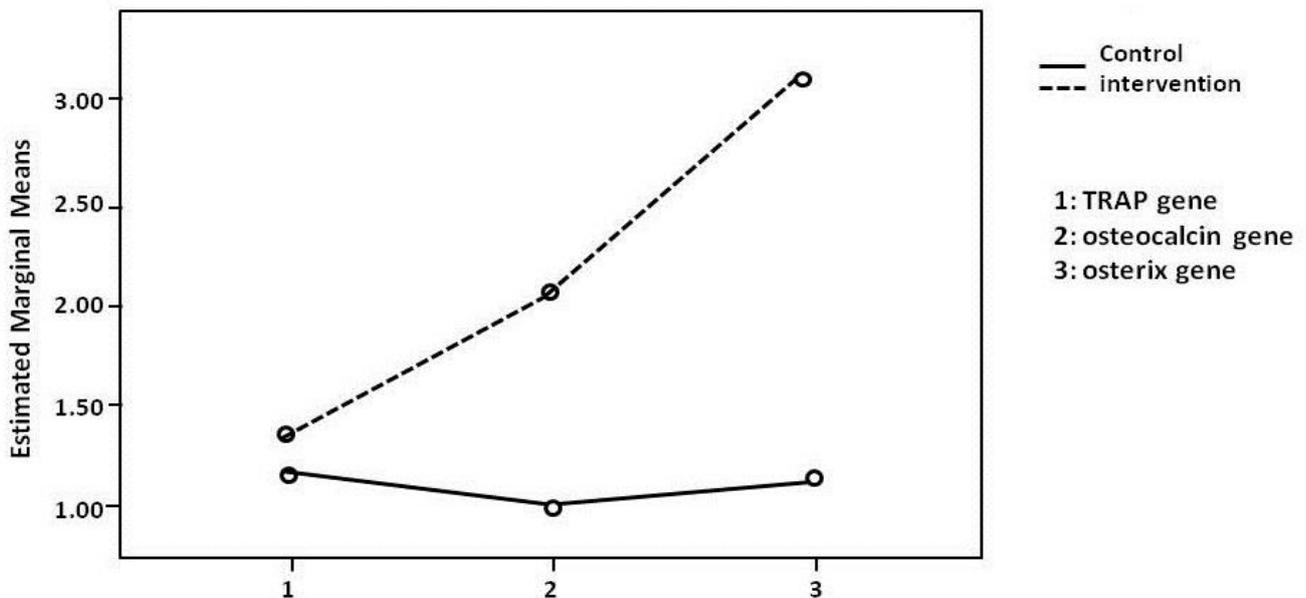

Figure 4. Expression levels of Osterix, TRAP and Osteocalcin genes in control and intervention groups.

Discussion

The main advantage of a three-dimensional environment over a two-dimensional environment is the interface decrease between cell culture system and cellular physiology.[15] Under two-dimensional conditions, the components of the extracellular matrix and cell-to-cell and cell-to-matrix communications that have an important role in differentiation, proliferation and function of the cells in vitro will be lost.[16] Some of the differences between the cells in the 3D and 2D environments include differences in sensitivity to medications, apoptosis, longevity, expression of genes, expression of proteins and differentiation.[3] In addition, it has been shown that when cells grow on a basement membrane such as a gel, a close cooperation will be created in the signaling pathways.[17]

In the present study, stem cells isolated from the Wharton jelly of human umbilical cord were cultured on 3D scaffolds in the presence and absence of PRP and their differentiation into osteoblasts was investigated by determining the expression of osteocalcin and osterix genes. The obtained data showed that the presence of PRP resulted in an increase in the differentiation of fetal MSCs into osteoblasts on 3D PCL/HA scaffolds. This finding is consistent with the results of studies carried out by Kasten in 2006, in which PRP resulted in an increase in proliferation, although its osteogenic properties on calcium-phosphate scaffolds were poor.[18] In another study, Rabies and Wang showed that statins result in induction and acceleration of the synthesis of localized bone, resulting in early expression of growth factors involved in angiogenesis, differentiation of osseous cells and osteogenesis.[19] Other studies have shown that the high concentration of platelets might be a determining factor for bone regeneration.[20,21] In a study by Baba in 2012, differentiation of osteoblasts from fetal umbilical cord stem cells was evaluated in vitro on hydroxyapatite scaffolds. The results showed an increase in differentiation.[22] Another study showed that PRP can promote osteogenesis in long term.[23]

Contrary to the results of the present study, a review study by Del Fabbro showed no consensus in relation to the advantages of the use of different concentrations of platelets in periodontal surgeries; furthermore, it was reported that the role of platelets in controlling the differentiation, function and apoptosis has not been fully elucidated.[10] A study by Kasten in 2008 showed that PRP had no effect on the osteogenic differentiation of mesenchymal cells.[24] In a study by Hernandez-Fernandez in 2013, too, there were no histological and radiographic evidence on the increase in osteogenesis in distraction osteogenesis in the presence of PRP during the initial phase.[25] In studies by Kon in 2010 [26] and Giovanni in 2011,[27] the presence of PRP was reported to have no relationship with bone regeneration.[24,28] It appears the discrepancies in the results might be attributed to differences in the concentrations of growth factors in PRP.[29]

Hsu[30] evaluated the opinion that PRP can prevent bone regeneration, reporting that PRP contains thrimbospondin-1 (TSP-1) which is a factor that inhibits angiogenesis, and PRP inhibits cellular proliferation at high concentrations. Since the results of the present study were contrary to those of the study above, it appears the discrepancy might be attributed to the type of the stem cells and the culture media used.

In the present study, differentiation of fetal stem cells into osteoclasts was evaluated by determining the expression of TRAP gene and it was shown that the presence of PRP did not result in an increase in differentiation of mesenchymal stem cells of the human umbilical cord into osteoclasts, consistent with the results of a study by OGINO et al.,[31] who showed that this might be attributed, to some extent, to the effect of PRP on an increase in the synthesis of osteoprotegerin. Another hypothesis might be the suppression of proliferation of macrophages; in this context, PRP can inhibit the proliferation of human macrophages in the culture medium.[32]

Contrary to the results of the present study, some in vitro studies have shown that isolated platelets result in an increase in or even induction of osteoclastogenesis.[33] The discrepancy in the results might be attributed to differences in the cell types and culture media used.

The final comparisons carried out between the effects of PRP on the expression of the three genes showed a significant difference between the expression of osterix and osteocalcin genes ($P<0.01$ and $P<0.05$, respectively). It appears when the megakaryocytes inhibit the differentiation of osteoclasts but increase the differentiation of osteoblasts, resulting in an increase in bone mass.[34]

In addition, it should be pointed out that, in models with excessive expression of TGF-β in vivo, the role of this agent in the differentiation of osteocytes has been reported to be unimportant; however, surprisingly no osteoblastic defects were detected. Excessive expression of TGF-β specific for osteoblasts in rats resulted in expression activation of osteoclasts and the final loss of a large amount of bone during turnover.[35] Studies have shown that prepared serum resulted in an increase in osteoblastogenesis; however, pure platelets result in a decrease in osteoblastogenesis.[36-39] The major difference is that in studies demonstrating osteoclastogenesis decrease, the prepared platelets contained serum components; however, in studies showing an increase in osteoclastogenesis, these bioactive molecules were not present to affect cellular function.[40] A study showed that the presence of PRP resulted in an increase in osteoblast and osteoclast counts at the graft site after one month. However, the number of osteoclasts and osteoblasts in the tissue in the bone graft site without PRP were similar to the presence of PRP in two months or more; therefore, the half-life of PRP appears to be short.[41]

Conclusion

According to the results of the present study, it can be concluded that the presence of PRP had no effect on the expression of TRAP gene. In relation with osterix gene, the presence of PRP with or without osteoblastogenic

factor resulted in an increase in the expression of the gene. Evaluation of the expression of osteocalcin showed that in the absence of osteoblastogenic factor, PRP resulted in an increase in the expression of this gene where the effect was not significant in the presence of osteoblastogenic factor. Overall, the results showed that PRP resulted in an increase in differentiation into osteoblasts, with no significant effect on osteoclasts.

Acknowledgment

Conflict of interests

The authors claim that there is no conflict of interest.

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