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Evaluation of the Healing of Critical Bone Defects Treated with Nanogel Scaffold, Platelet-Rich Plasma and Freeze Dried Bone Allografs Alone or in Combination in the Rabbit

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Abstract: Reconstruction of critical bone defects is one of the most important issues in medical science where the use of materials with properties osteoconduction, osteoinduction and osteogenic needs. In some studies, the posetive effect of platelet rich plasma and other studies have reported no effect on the healing of bone defects. Current study also aimed to assess the restoration of critical bone defects treated with platelet rich plasma, nanogel bone scaffold and allograft alone an in combination. In this experimental study, 50 adult New Zealand white rabbits were randomly divided in five groups. After general anesthesia, under sterile conditions to help trephin drill hole size 8×12 mm in the femor of lateral condyl each of the animals created. In control group (G1) the defect was filled by blood clot only. In G2, G3 and G4, inside cavity were replaced nanogel scaffold, nanogel scaffold+PRP and nanogel scaffold +PRP, +FDBA, respectively. At weeks 2, 4 and 8 after surgery, three animals from each group were randomly selected and then sampling of the defect, the bone repair techniques using histological and histomorphometric parameters. Data were analyzed by Kruskal-Wallis and Mann-Whitney U tests and $p>0.05$ was considered significant. The minimum amount of bone formation between groups was belonged to the control group. The mean bone parameters were significantly different in the second week ($p<0.05$) and the average of all bone parameters in the G1 and G2 were zero in this week. In the fourth week of the fourth group, mean resorption surface was (83.04±2.65) and mean bone volume was (81.83±4.60). The mean bone parameters were significantly different in the fourth week ($p<0.05$). In the eighth week of the fourth group, mean resoption surface was (81.61±1.81) and mean bone volume was (83.20±1.40). The mean bone parameters were significantly different in the eitghth week ($p<0.05$). The results of this study showed that the bone grafts combined with PRP compared with none combining them with PRP increases the healing rate of critical bone defects. Also, healing rate of bone defects using allograft is more than with nanogel scaffold.

Key words: Bone healing, platelet rich plasma, bone allograft, rabbit, nanogel scaffold

INTRODUCTION

The main role played by bones is to provide a strong and secure framework for the body to protect and maintain soft tissues such as brain, heart, lung and so on. Moreover, the tissue is of pivotal importance in body movement, mechanical protection and in the process of hematopoiesis (Junqueira and Carneiro, 2005). Further, the tissue regulates the concentrations of ions and other mineral compounds in the body fluids (Ross and Pawlina, 2006). There is a close relationship between this tissue and immunsystem; lymphocytes B which have the duty of supplying the body with different antibodies, are produced by bone marrow. Accordingly, it seems necessary to pay enough deserved attention to care such an important tissue. Fortunately, bones have a dynamic structure which makes them able to repair themselves following a damage or fracture (Ross and Pawlina, 2006). For this reason, bones are categorized in the group of self-healing tissues (Thomson *et al.,* 1998). The capability

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of a bone to repair itself is reliant on the severity of the damage. In cases that damage is of high severity and a considerable amount of bone and associated muscles, blood vessels and neurons are destroyed or lost (critical defect), the damage would be improbable to be restored thoroughly and there would remain a minimal discontinuity in shape of a fibrous and loose tissue at the damaged regions. Critical damage of a bone is the minimum incurable damage that cannot be spontaneously restored during the life of a living creature. In a more technical expression, critical damage of a bone refers to some types of damage which its repair is lower than ten % throughout the life of a living creature (Hollinger and Kleinschmidt, 1990). According to the studies carried out in this area, damage to rabbits' bone should be regarded as a critical one if its diameter is more than 4 mm and its depth is higher than 6 mm (An and Freidman, 1998). Considering the fact that millions of individuals worldwide are suffering from bone damages caused by various causes such as traffic accidents, tumors, collisions and so on and most of them might lose their organs or in severe cases, even their lives, it seems to be a great challenge to overcome such damages. There have been conducted a high volume studies so far on treating and restoring such damages in particular in the field of orthopedic and maxillofacial surgery (Rodriguez *et al*., 2014). A wide range of treatment approaches has been employed in this regard, varying from primary metal implants to advanced techniques such as gene therapy and growth factors release. In recent years, there has been an interest of using Platelet Rich Plasma (PRP) in this respect. Such a compound is commonly obtained from the own blood of the injured person who suffers from bone damages and contains growth factors which play a pivotal role in regulating the growth of damaged tissues. PDGF, TGFβ, EGF, VEGF and IGF1 are some of these factors (Sanchez *et al*., 2003). The effectiveness of a combination of PRB and grafts in lowering the age of majority and treating tissue damages has been demonstrated by several studies (Marx *et al.,* 1998). However, there are some studies as well that have rejected such outcomes (Badr *et al.,* 2010). Accordingly, the present study was set to investigate and compare the effectiveness of PRP, nanogel scaffolds, bone allograft and their combination in repairing critical damages.

MATERIALS AND METHODS

Preparing PRP: Before the surgery, 6-7 mL blood was taken from femoral artery of the animals using a ten cc syringe and preserved in silicone containers containing 8.3% sodium citrate solution for protecting the blood from clotting (one cc 8.3% sodium citrate for each 9 cc of blood) (Dallari *et al.,* 2006). Next, the sampled blood was centrifuged at 1000 rpm for 5 min. using a special sampler, plasma was separated from red blood cells from buffy coat area and the red blood cells were disposed. Then, the separated plasma was centrifuged again but at a higher speed (1900 rpm) and for a longer period of time (15 min) (Dallari *et al.,* 2006). The number of platelets in the blood samples taken from rabbits was initially counted as $323.81\pm73.5\times10^3$ cells per μL of blood while platelet concentration in the processed PRP was equal to $1200.03\pm253.88\times10^3$ cell μL. before placing the PRP, one cc chloride calcium 0.025 M per nine cc of PRP was added to the damaged area in order for activating platelets.

Bone allografts (FDBA): Similar to auto graft, allografts are also acquired from the body of a living creature from the same species but other than the one that will use it. These materials are normally stored in a bone bank (Katuri *et al.,* 2013). These types of graft are prepared by a process known as the freeze-drying in which fluids are separated from bones by applying a dry, cold stream, then free of liquid bones are packaged in a vacuum condition and stored at the room temperature. Storing at the room temperature cause the bones to lose osteoarthritis transgenic cells and retain osteoconductive characteristics. Because of denaturing of bone proteins which seems to be inevitable, reconstruction of blood vessels may be retarded (Lasanianos *et al.,* 2008).

Nano gel scaffold: Bone scaffolds are a new group of material in engineering sciences which are mostly utilized for restoring bone damages. Having an osteoconductive characteristic, bone scaffolds are built in various shapes and being studied extensively. In the present study, we used a Nano gel scaffold which was built from rabbit's bones. The scaffold had a Nano structure which contained such ingredients as gelatin and biological glue and was manufactured using the freeze-drying process. In fact, the structure was obtained by sandwiching a layer of spongy tissue between two layers of dense bone tissue.

Animal model and surgery: In the present study, a total number of 50 mature male rabbits of the New Zealand race with ages ranged from 6-9 months and weight of 2.5-3.5 kg was investigated. Preserving in a separate cage with a standard air condition and full access to enough food and water, rabbits were randomly categorized into five equal groups; each contained ten rabbits, nine ones for experiments and the last one as the reserve. The rabbits were anesthetized by intra-muscular injection of ketamine hydrochloride (44 mg kg^{-1}) and xylazine (3 mg kg^{-1}). In

the next step, the thigh hair of rabbits was shaved on one side to below the knee and the skin of the femur external condyle was disinfected with a butadiene solution. Then, the exact place of the surgery was covered by sterile clothes. Using a No. 15 scalpel blade this area was cut 5 cm. A hole of 8 mm diameter and 12 mm depth was created on the femur external condyle using a surgical microdrill with Trfayn bit. According to the group of the study, different approaches were employed for filling in the holes; In group 1, the control group, the holes were filled in by clotted blood; a Nano gel scaffold with dimensions of 5×2 mm was used in the group 2; the holes in groups 3 of rabbits were filled by the mentioned Nano gel scaffold and 1 cc PRP; the Nano gel scaffold alongside 1 cc PRP and 1 cc allograft were used for treating the holes created in group 4 of rabbits.

At the end of surgery, periosteum of the bones was brought back to their initial places; muscles and fascia were repaired using a 0.3 chromic thread. The skins also were stitched using a 0.3 nylon thread. After implementing surgery and for any infection to be prevented, cefazolin was intramuscularly injected at the dosage of 100 mg kg^{-1} to all animals for 24 h (Dallari *et al.,* 2006). Furthermore, in order to relieve pain after the surgery, a suppository of acetaminophen 125 was given to each rabbit. In the weeks 2, 4 and 8 after the surgery, three rabbits were randomly selected from each group and killed by a high dose of chloride potassium, then several samples were taken from the damaged area using Trfayn 10.

Histology and histomorphometry: The samples taken from damaged bones were immediately transported to the containers containing 10% formalin and remained into them for at least 15 days to be stabilized. In the next step, samples were emerged in 18% EDTA for 3 weeks to be decalcified (Neovius and Engstrand, 2010). After this step, samples were placed in containers of formalin for 15 days to be stabilized again. Lastly and after some preparation steps, a paraffin block was provided for each rabbit, several samples with a diameter of 6 μm were serially cut using a Microtome device and placed on the glass slides. The samples were stained using such protocols as trichrome Mason's and hematoxylin and eosin staining were assessed under an optical microscope. Several images were taken from these samples using a digital camera (Olympus DP 10, Olympus Optical, Tokyo, Japan) which had been connected to the optical microscope. Utilizing an ocular graticule (ZEISS, 1kp1-w12.5×18 model), scale ruler and the optical microscope with a magnification of 40 times the samples were investigated in details and several indices such as bone volume, osteoid volume and surface area were measured and recorded (Goss, 2009).

Statistical analysis: The data of the present study were analyzed using Kruskal Wallis and Mann-Whitney tests performed by SPSS Software package Version 20 developed by IBM. All tests were conducted at the 0.05 level of significance.

RESULTS AND DISCUSSION

At the end of week two, the results of staining demonstrated that some cells in group one (control) and group two were inflamed. Moreover, it was observed that osteoblasts were proliferating and loose connective tissues were emerging at this stage. Interestingly, the activity of osteoblasts was higher among samples of group three in comparison with those of two previous groups and a higher amount of blood vessels, moreover, was observed in this group, emphasizing on a higher rate of ossification and collagen formation in this group compared with two previous groups. The activity of osteoblasts among samples taken from the group four of rabbits was so high that osteoid had been formed in some regions (Fig. 1). At the end of week 4, the number of cells participated in the inflammation process was significantly reduced among both groups one and two. Moreover, at this week, fibro connective tissues had a higher growth and a more regular tissue structure was observed than what had been observed in previous weeks. The osteoid formation process was progressing as well. In group three, osteoblasts found more regularity and the number of spicules were increasing. In group four, at the end of this week, spicules were being connected to each other and tissue found a trabecular appearance (Fig. 2). At the end of week 8, the process of bone repairmen was improved in all groups and osteoid tissues were being mineralized in all groups, especially the fourth one. At the end of this week, moreover, the number of spicules was being increased. The tissues formed in samples taken from group three of rabbits found a trabecular appearance and osteocytes alongside lacunas were observed in some of trabeculae. The number of trabeculae in samples taken from group four of rabbits was higher than those of other groups and, more importantly, bone marrow tissue was observed in these samples at this period of time (Fig. 3). Statistical analyses revealed that the mean values of all indices obtained from group four of samples were higher compared with those of other groups. As evident in Fig. 4, the Chi-square test showed a significant difference between the groups in terms of these indices ($p<0.05$). Moreover, the mean values of all bone-related indices in group one (control) and group two (Nano gel scaffold) in the first week were equal to zero (Fig. 4). At the end of the 4th week, the mean value of absorptive surface area of samples from group four was 83.04±2.65

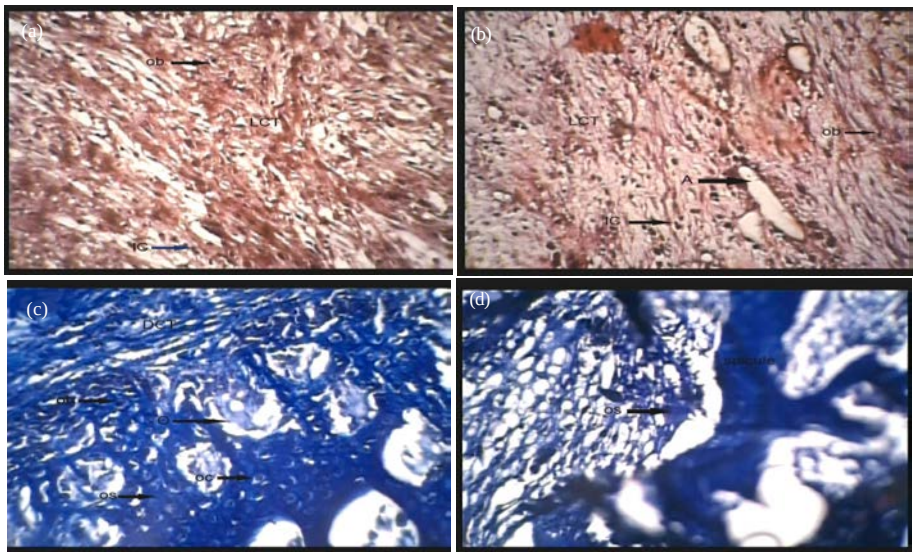

Fig. 1: Histopathological changes of groups at the end of week 2; staining by three chrome mason:a and b) The associated with groups one and two, respectively. The presence of immunocells is evident which are differentiated with a large dark nucleus. The presence of these cells is indicative of inflammation. Moreover, osteoblast cells which have a smaller nucleus and are proliferating. Further, the loose connective tissues are somewhat formed; c) Group three, the blue regions are indicative of formation of connective tissue (beginning of repair) which are full of collagens fibers (DCT) as well as osteoblast cells. Moreover, it seems that the activity of osteoblast cells is so high that osteoid is being formed in some regions; d) Group four, osteoid tissue is now formed and osteoblasts are transforming in osteocytes (Magnification 40x)

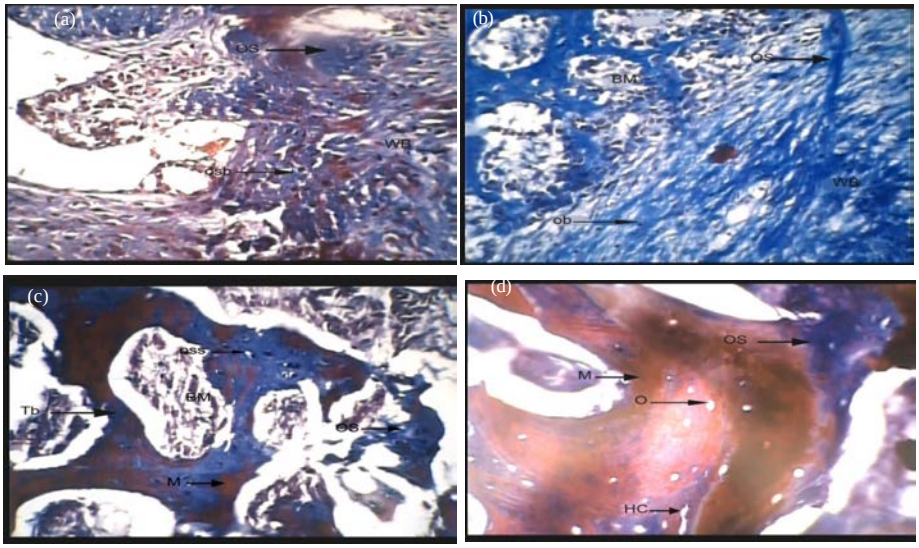

Fig. 2: Histopathological changes of groups at the end of week 4; staining by three chrome mason: a) Group one there is no sign of inflammation, connective tissues are increased, osteoid volume is also increased and some spicules are formed; b) There is no inflammation, spicules are forming and osteoid volume is enlarging; c) Group three that trabeculae are progressing and in some regions osteoid tissue are mineralizing and in some other regions bone marrow is observed; d) Group four, the remodeling process of the bone is obviously improved so that most of it is mineralized (red regions), however, osteoid volume is decreased (blue regions), Osteocytes (O) and Harversian Canal (HC) (Magnification 40x)

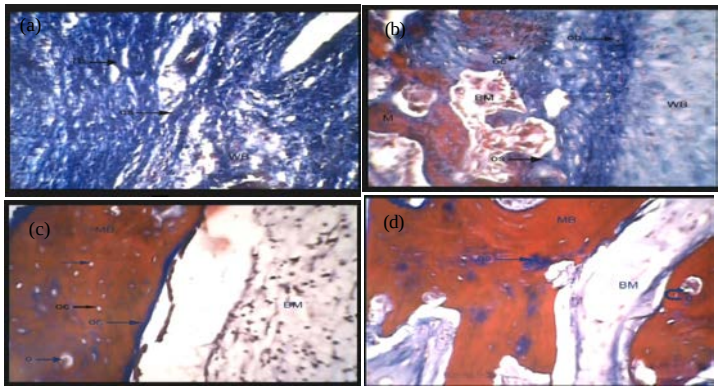

Fig. 3: Histopathological changes of groups at the end of week 8; staining by three chrome mason: a) Group 1 it can be seen that the number of Trabeculae (Tb) are increased and osteoid is Minimized (M) in some regions (regions in green); b) Group two it can be deduced that the number spicules are increasing, Trabeculae (Tb) are forming; c) Majority of osteoid tissues are mineralized, the activity of osteoblasts is decreased and transformed in osteocytes (oc); d) The remodeling process is considerable progressed because of the increase in trabeculae, moreover, osteocytes and osteoid are evident (Magnification 40x)

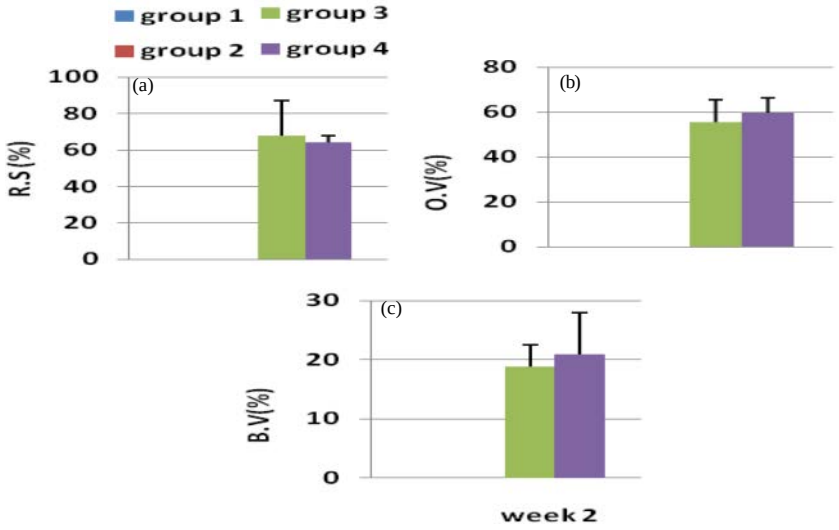

Fig. 4: The indices values for all groups; the RS chart represents the %age of bone absorptive surface area for all groups which was higher in group three (68%) in comparison with that of group four (65%). The OV chart represent the percentage of osteoid volume which was higher in group four (60%) compared whit that of group three (55%). Finally, the BV chart represents the percentage of bone volume which was higher in group four (22%) compared with that of group three (18%)

and mean value of bone volume was 81.83±4.6 which were significantly higher than the values obtained for other groups. Furthermore, at the end of the 4th week, the mean value of osteoid volume was decreased for both groups three and four which was because of the increase in mineralization of the osteoid tissues (Fig. 5). At the end of week 8, the mean value of RS in samples of group four was 81.66±1.81 and the mean value of BV was 83.20±1.40 (Fig. 6). In addition, the differences among all groups at the end of this period of time were significant ($p<0.05$).

The present study was conducted to assess the effectiveness of several approaches in repairing bones with critical damages. The approaches were as follows:

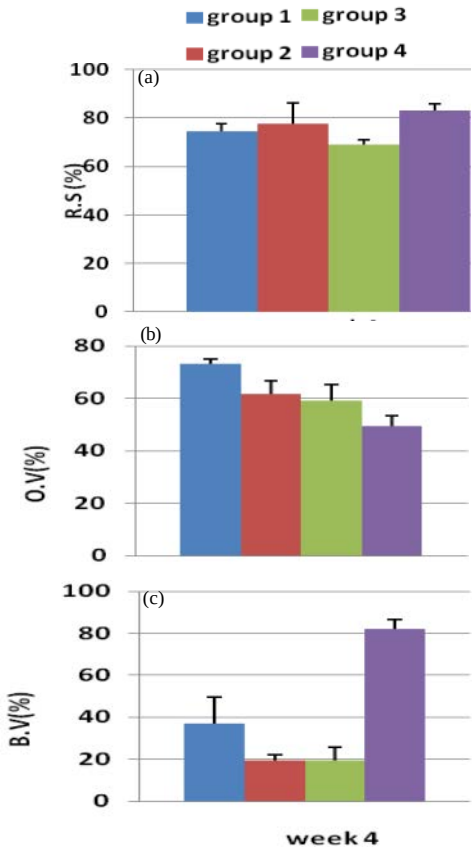

Fig. 5: The indices values for all groups at the end of week four; it is evident from this chart that group four had the highest RS index (83%), likewise, this group had the most pleasant outcome in terms of BV index. Moreover, at the end of this period of time (week four), OV percentage was at the most in group one (72%). There were significant differences among groups ($p<0.05$) and the repair of bone was higher in samples of group four compared with those obtained from other groups

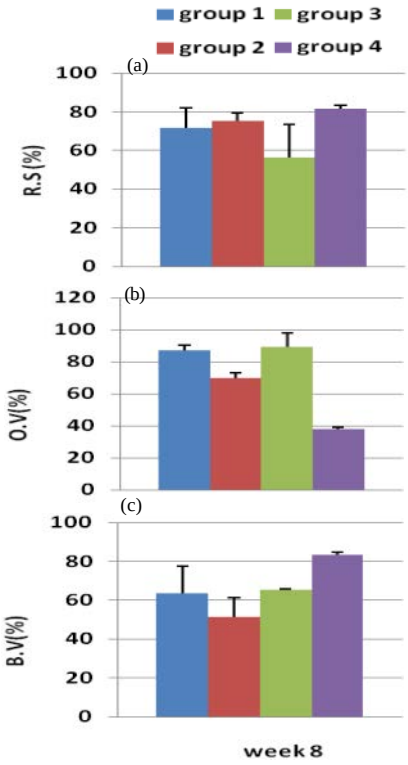

Fig. 6: The value of indices at the end of week 8; this chart demonstrates that the differences among groups were significant ($p<0.05$) and the repair of bone was higher in group four than those of other groups

the use Nano gel scaffold alone, (Junqueira and Carneiro, 2005) the use of Nano gel scaffold alongside PRP, (Ross and Pawlina, 2006) the use of Nano gel scaffold, PRP and bone graft. The results showed that the ability of these approaches in repairing and restoring damaged bones were significantly different over time. However, there was not observed any significant difference between control group and bones treated by Nano gel scaffold alone (group two) which was in contradiction with most of previous studies. Further, significant differences were observed between the mean values of bone-related indices of groups two and three, suggesting that PRP had a remarkable effect on the bone repair process. It has been illustrated by several studies that PRP alongside bone or biologic grafts is able to enhance the pace of bone growth, development of blood vessels and differentiation of osteoblasts. Such important factors as TGFβ and PDGF released by platelets are of high importance in this regard (Schlegel *et al*., 2004). In addition, studies have demonstrated that PRP is very effective in improving the proliferation of endothelial cells (Frechette *et al*., 2005), immature osteoblast cells (Frechette *et al*., 2005) and mature osteoblast (Ogino *et al*., 2006). However, it should be noted that there have been several studies that reported PRP has no considerable effect on repair of a damaged bone (Abedi *et al*., 2012). By reviewing previous studies carried out in this area, it becomes appear that there are conflicting results about the effect of PRP on bone growth. Use of different methods for extracting PRP can be a possible explanation for this discrepancy. Some studies have stressed on the importance of the method using which PRP is extracted (Nagata *et al*., 2009; Abedi *et al*., 2012). In addition to the extraction method, another important factor about PRP that should be taken into

account is the way of activating platelets in PRP. In the present study, chloride calcium was the only matter used for doing so, while in the similar study carried by Dalari et al (Dallari *et al.,* 2006) chloride calcium as well as bovine thrombin were applied in this regard. It should be stressed that studies reported that using chloride calcium alone is the most effective one (Messora *et al.,* 2008). It is worth mentioning that the use of bovine thrombin can create coagulation disorders, so it is prudent to avoid using such a compound (Spero, 1993). The most appropriate alternative of bovine thrombin is the thrombin obtained from the own blood of the patient. However, thrombin extraction using independent methods is difficult to do (Somer *et al.,* 2006). In this respect, a conventional method is to extract thrombin from PRP, but it can reduce the effectiveness of PRP (Somer *et al.,* 2006). In the present study, it was observed that there was a significant difference between the mean values of bone volume index of two groups three and four, emphasizing on the role of allograft in enhancing bone repair process. In fact, the effectiveness of allografts in this regard has been stressed by many studies (Aghaloo *et al.,* 2005). In some studies it has been reported that the success rate of allograft transplantation surgery is about 61.5% while the probability of such a treatment to be unsuccessful is only about 11% (Nishida and Shimamura, 2008). If an allograft does not have some key characteristics such as osteocondutivity and osteogenicity, it would only act as a scaffold and would not affect the pace of bone growth (Reikeras *et al.,* 2010). Although, it was reported by some studies that using allografts such as FDBA can result in immunological reactions (Turner and Mellonig, 1981), there was not observed such a thing in the present study.

CONCLUSION

In conclusion, applying a combination of PRP and bone grafts can be effective in enhancing the repair of bones suffering from critical damages. Moreover, bone allograft is a better choice than Nano gel scaffold in repairing damaged bones.

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