

# In Vivo Bone Formation Assessment Using PRP With Chitosan (Animal Comparative Study)

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## Abstract

Guided Bone Regeneration is a surgical treatment that involves the use of barrier membranes, particulate bone transplants, and/or bone substitutes. Sheep's weighted 20-30 kg were intubated and underwent general sedation with 0.03 mg / kg IM xylazine. The 8mL of blood was taken from external jugular vein of the sheep and immediately transferred into a citrated tube and transferred to centrifuge to prepare the PRP gel. The produced PRP was mixed with chitosan in 95:5 ratio. With a round bur and abundant irrigation, a 4-5 mm round osteotomy was formed in the pad of the maxilla with two in the right and two in the left side. Four equal defects were grafted with experimental material composed of mixed PRP and chitosan (95:5 ratio). After four weeks, the results show new bone formation around the defect side which was associated with interrupted fibrous connective tissue capsule for some of the samples with focal active osteoblasts. In ten weeks, the results show thick bones were newly formed at the defect side with remnants of chitosan observed at the site of bone. In conclusion: the present study revealed the combination of PRP and Chitosan accelerates the healing and regeneration of bones.

**Keywords:** Bone regeneration, Chitosan, PRP.

## INTRODUCTION

Guided bone regeneration is a common surgical method for increasing the quality and quantity of host bone in localized alveolar bone lesions. Allografts and autografts, xenografts, as well as alloplastic bone substitutes are some of the methods reported for increasing the rate of bone development and augmenting bone quantity.<sup>1</sup>

Tissue engineering relies on triggering a sequence of processes and cascades at a single site, that can result in the coordination and completion of integrated tissue creation. For the enhancement of bone regeneration, several biological techniques have been used, including the bone metabolism mediators, usage of growth and differentiation factors, attachment factors and extracellular matrix proteins.<sup>2</sup>

A biological mediators like polypeptide growth factors that can control cell differentiation, proliferation and chemotaxis among other things. Despite the fact that several recombinant or natural growth factors have been launched to promote bone formation, their clinical usage is restricted due to cost and

immunological issues. Their short lifespan and ineffective delivering to target cells are significant interest.<sup>3</sup>

Chitosan is a key component in both medicine and cuisine. It's a polysaccharide made up of glucosamine and N-acetylglucosamine copolymers that can be made by partially deacetylating crustacean shell chitin.<sup>4</sup>

Platelet rich plasma (PRP) is another simple and cost-effective technique to access high concentrations of growth factors to the healing of tissue and regeneration. It is a tiny amount of

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plasma with an autologous concentration of human platelets. It also contains TGF b1, TGF b2, epithelial growth factor and vascular endothelial growth factor, which are all important protein growth factors as well as cell adhesion molecules involving fibronectin, fibrin, and vitronectin are also present.<sup>5</sup>

Despite the fact that these growth factors are recognized to aid regeneration, but, it is still a pressing need for a formable and osteoconductive scaffold for regeneration of tissue which also serves as a vehicle for matrix components and growth factors. The effects of combining chitosan and PRP have been reported to increase the release of growth factors from PRP as well as the expression of glycoprotein IIIa in platelets.<sup>6</sup>

Due to the findings in impacts of PRP and Chitosan on regeneration of bone that growth factors are released from activated human platelets after chitosan stimulation, it has been suggested that chitosan might be utilized as a replacement for thrombin in PRP preparation.<sup>7</sup>

## METHOD

### Experimental Preparation

The chitosan CH powder {75-85% deacetylated chitin, poly (D-glucosamine)} will be provided from Sigma-Aldrich, USA and prepared according to the manufacturer instructions. The powder is white flaky and has a medium molecular weight (190,000-310,000 Dalton). The chitosan solution will be made via dissolving 1 g of chitosan powder (weighted in a digital weight balance) in 1000 mL of acetic acid (0.1 Mol/L) then, stirring and heating at about 55 C by keeping in an oven overnight to form a clear homogeneous 10 g L<sup>-1</sup> chitosan solution.

The solution will then be filtered through a paper filter to get rid of dust and insoluble impurities and will be used as a stock solution.

### Preparation of PRP

A citrated tube was used to draw the blood sample. After that, the sample tube was spun for 10 minutes at 2400 rpm in a conventional centrifuge to create platelet-poor plasma. The platelet-depleted plasma was drawn into a syringe with a long cannula and a second air-intake cannula. To concentrate the platelets, a second centrifugation (15 minutes at 3600 rpm) was used. A long cannula and an air-intake cannula were also used to absorb the second supernatant. The volume of supernatant was roughly 0.6–0.7 mL per 10 mL of blood: this was the PRP.<sup>8</sup>

### Mixture Preparation

The mixture was prepared by adding a chitosan solution to PRP solution in 5:95 volume/ volume ratio.

### Surgical Procedure

The sheep weighted 20-30kg were intubated and underwent general sedation with 0.03 mg / kg IM xylazine. The 8 mL

of blood was taken from external jugular vein of the sheep and immediately transferred in to citrated tube containing 2 mL sodium citrate and shaken to prevent coagulation of blood and transferred to centrifuge to prepare the PRP gel by placing in 2400 rpm for 10 minutes then the PRP was separated from RBC and PPP. The produced PRP was mixed with chitosan in 95:5 ratio and part of it was separately used as implantation material then the surgical area was prepared by disinfectant solution and anesthetized by local anesthesia lidocaine 2 mg/kg by infiltration technique. An appropriate incision was made on the pad of maxilla and mucogingival flap was reflected on mucogingival junction. A 4-5 mm round osteotomy was made in the pad of maxilla with a round bur with copious irrigation two in right and two in left side. With a round bur and abundant irrigation, a 4-5 mm round osteotomy was formed in the pad of the maxilla with two in the right and two in the left side. Four equal flaws were grafted with a mixture of PRP and chitosan as an experimental material (95:5 ratio), PRP gel, chitosan alone and one of the defects was used as control. To give early healing stability for the PRP and chitosan and to prevent tissue infiltration into the defects through their superior apertures, all flaws were sealed with a non-resorbable membrane (Cenobone 20\*30mm). 3.0 silk sutures were used to seal the wound. To avoid postoperative infection in the animals, kanacin solution (1 mL/25kg) was given. Each animal is kept in a 25-degree Celsius incubator until it regains consciousness following surgery. Separate cages are used to hold the animals.

### Tissue Processing and Histological Evaluation

By the fourth and tenth weeks, the sheep were dead. Without infringing on the grafted portions, the entire bone around the grafted area was removed. The specimens are fixed in a 4 percent formalin solution for 48 hours before being decalcified in 10% formic acid. The specimens are arranged so that both the healthy bone and the surgical flaws are visible in the same area. The histological specimens are prepared as normal with hematoxylin and eosin at a thickness of 5 mm. A light microscope is used to examine the histological sections.

## Results

### Histologic Result After Four Weeks

#### 1. Mix (chitosan and PRP)

The histologic result showed evidence of new bone formation around the defect side which was associated with interrupted fibrous connective tissue capsule for some of the samples with focal active osteoblasts. Little active fibroblast stroma with no fibrous stroma had been recorded. No angiogenesis with absent necrotic nor inflammatory cells had been noticed. No remnants of chitosan were

detected. The large lacunae with osteocytes that define newly produced bone, neither osteoclastic resorption nor remodeling process had been clear. Figure (1) shows new bone formation and fibrous stroma.

## 2. PRP

The defect areas were covered by new bone formation with partially formed connective tissue capsules between

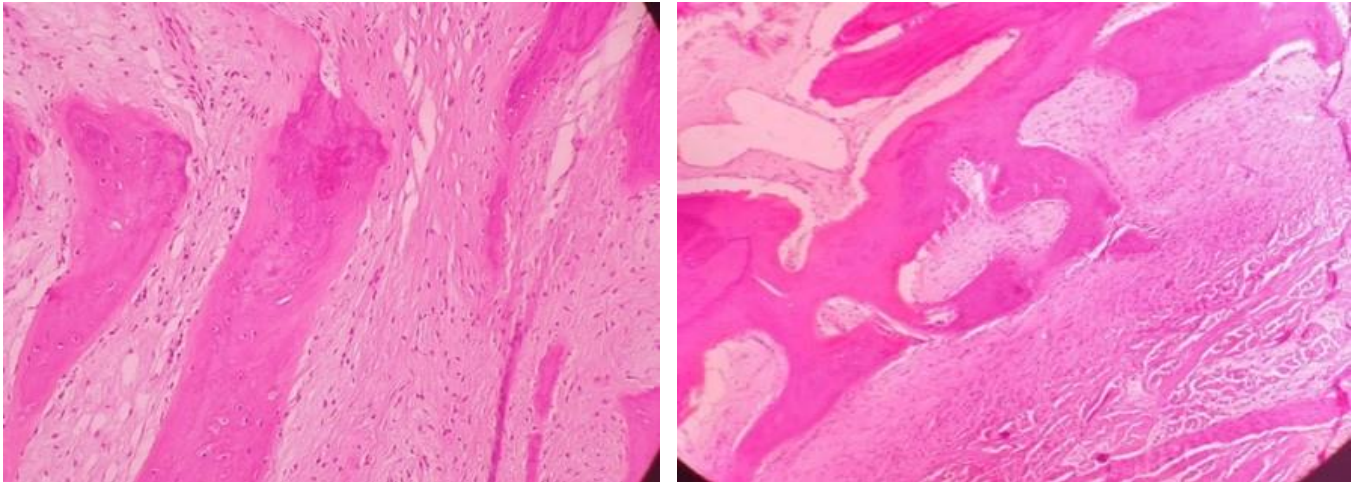


**Fig. 1:** New bone formation and fibrous stroma (X 10)

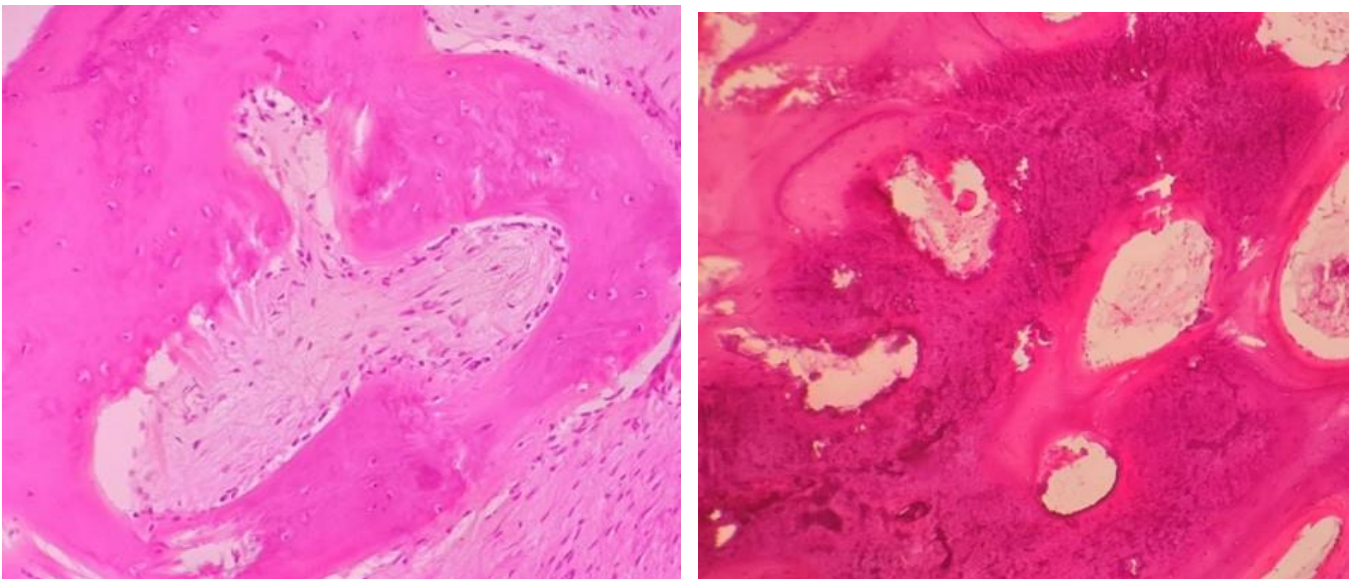
the bone trabeculae which were associated with a focal amount of osteoblastic activity adjacent to the newly formed bone. Active fibroblast stroma with fibrous stroma had been recorded without any inflammatory reaction. No angiogenesis with absent necrotic nor inflammatory cells had been noticed. No remnants of chitosan were detected. Wide lacunae containing osteocytes defined newly produced bone, neither osteoclastic resorption nor remodeling process had been clear. Figure (2) shows fibrous stroma and reactive bone in different amplification degree.

## 3. Chitosan

The histologic section showed a large amount of chitosan remnants surrounding the newly formed bone which were associated with a mostly complete capsule of connective tissue with little focal amount of active osteoblast and little fibrous stroma without active fibroblasts. Inflammatory



**Fig. 2:** (A) Fibrous stroma and reactive bone (20X); (B) Fibrous stroma and reactive bone ( X10 )



**Fig. 3:** (A) Osteoblastic rimming (20X); (B) Remnant of chitosan (10X)

reactions at the defect side were not detected. Osteocytic lacunae were observed around the newly formed bone. No angiogenesis with absent necrotic nor inflammatory cells had been noticed. Neither osteoclastic resorption nor remodeling process had been clear. Figure 3(A) shows impaired bone and figure 3 (B) shows healing of bone and remnant of chitosan.

#### 4. Control

Evidence of newly formed bones were detected with very little of a thin plate of connective tissue capsule somewhat surrounded by active osteoblasts. Very little of active fibroblasts without fibrous stroma without any inflammation. Osteocytic lacunae were observed around the newly formed bone. No angiogenesis with absent necrotic nor inflammatory cells had been noticed. Neither osteoclastic resorption nor remodeling process had been clear. Figure (4) shows Osteoclast under (20X) amplification.

### Histologic Result After 10 Weeks

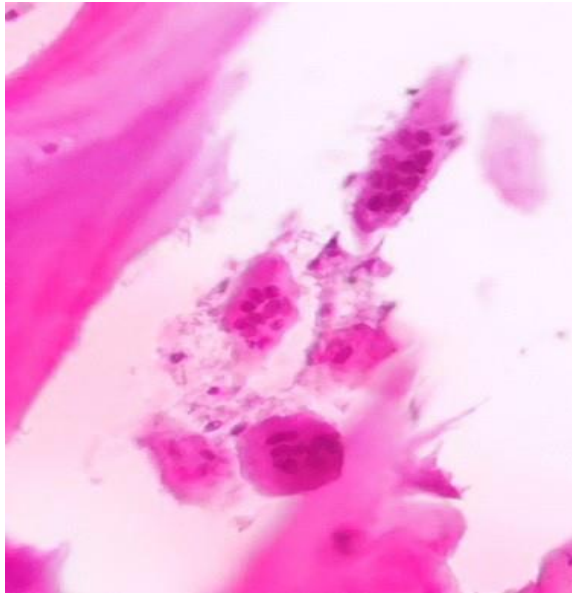
#### 1. Mix (chitosan and PRP)

Thick bones were newly formed in figure (5) at the defect side with remnants of chitosan observed at the site of bone which were covered by thick connective tissue capsules with a focal amount of active osteoblasts in the periphery of the defect area with little fibrous stroma in between trabeculae observed without inflammatory response or active fibroblasts, and the newly formed bone composed of osteocytes lacunae and osteocyte cells. No angiogenesis with absent necrotic nor inflammatory cells had been noticed. Neither osteoclastic resorption nor remodeling process had been clear.

#### 2. PRP

Defect is completely occupied by a thick bone trabeculae with focal osteoblastic cells. Thick connective tissue capsules

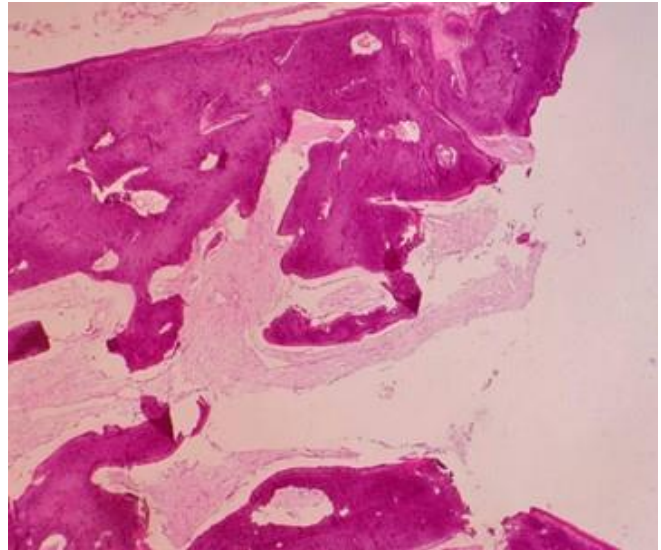
surrounding the new bone with numerous fibrous stroma in figure (6) between the dense bony trabeculae without inflammatory reaction and without active fibroblasts. No angiogenesis with absent necrotic nor inflammatory cells had been noticed. Neither osteoclastic resorption nor remodeling process had been clear.



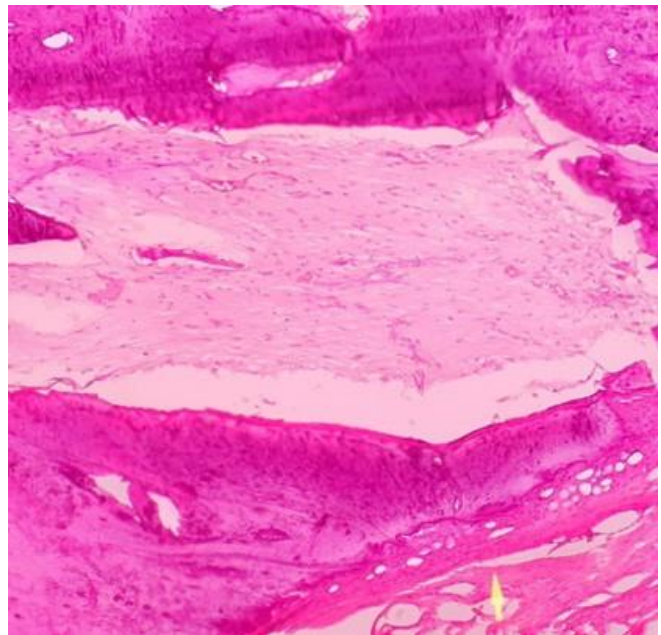
**Fig. 4:** Osteoclast (20X)

### 3. Chitosan

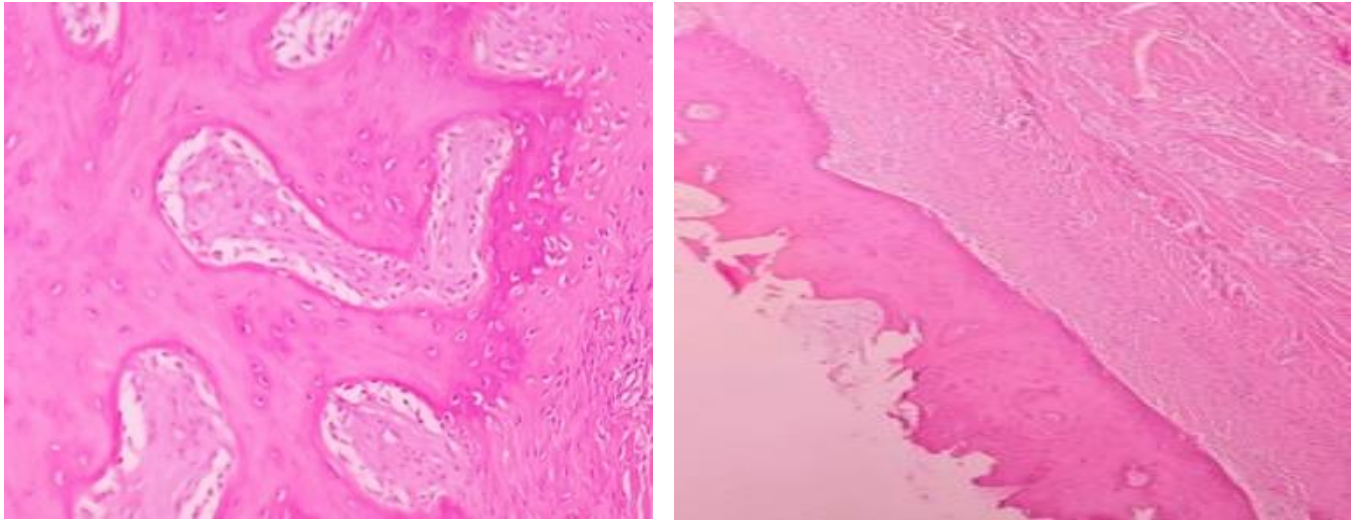
Little remnant of chitosan was detectable homogeneously around the newly formed bone at the defect side which were covered by thick connective tissue capsule with bone encircled by active osteoblast and osteoblastic rimming were observed with thin fibrous stroma without inflammatory response. Thick bone contained osteocyte lacunae and osteocyte cells. Figure (7) shows Osteoblastic rimming and formation of Fibrous capsule around bone.



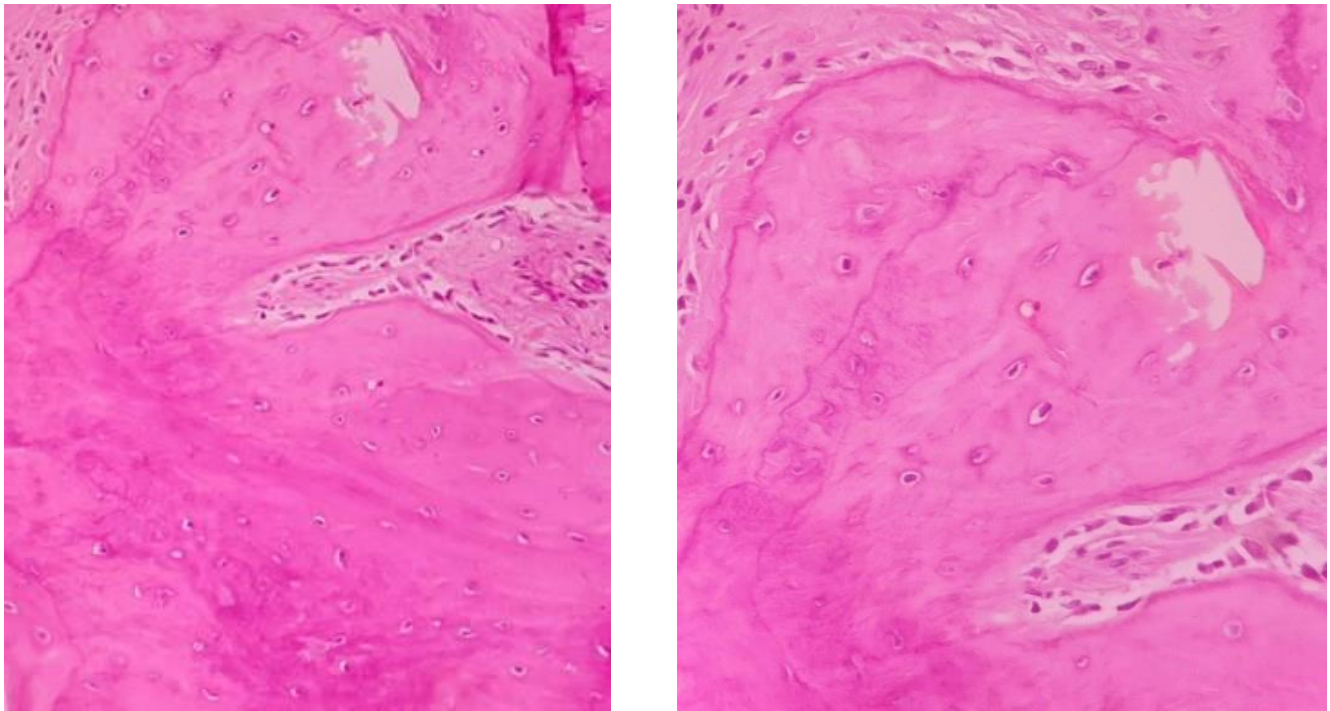
**Fig. 5:** Thick bone (40X)



**Fig. 6:** Fibrous stroma (10X)



**Fig. 7:** (A) Osteoblastic rimming (20X); (B) Fibrous capsule around bone (10X)



**Fig. 8:** (A) Woven bone (20X); (B) Woven bone (40X).

#### 4. Control

Woven bone formation with thick fibrous capsule surrounds the defect area noted with little active osteoblast observed with detection of thick fibrous stroma around new bone trabeculae in the side of bone regeneration. There was no evidence of inflammatory response and there was no necrotic tissue. Figure (8) shows woven bone in different amplification degree.

#### Discussion

Inflammation, proliferation, and tissue remodeling are three stages of the process of healing that occur after tissue damage that is as similar to its native form as feasible. When platelets come into touch with exposed collagen, they clump together and release clotting factors, leading to the formation of a fibrin clot at the injury site. The fibrin clot act as a provisional matrix and sets the stage for the subsequent events of healing. Along with platelets, inflammatory cells arrive at the injury

site and release growth factors, which are important signals. The fibroblast is a connective tissue cell that helps to repair tissue damage by forming collagen. Collagen is the most important component of extracellular tissue, as it provides strength and support.<sup>9</sup>

PRP is a concentrated platelet with a high concentration of autologous growth factor in a little volume of plasma.<sup>10</sup> Because it offers a variety of bone remodeling effects, it's commonly employed in clinical dental procedures like augmentation, sinus floor elevation, maxillary cleft repair and alveolar ridge mandibular reconstruction. It can also be used to treat periodontal disease since it promotes bone repair.<sup>11</sup>

It is widely used because it is inexpensive and simple to prepare, and it contains a large number of growth factors such as (PDGF $\alpha$ , PDGF $\beta$ , PDGF  $\gamma$ , TGF  $\beta$ 1, TGF $\beta$ 2, epithelial growth factor and vascular epithelial growth factor), as well as cell adhesion molecules involving fibrin, fibronectin, and vitronectin.<sup>12</sup> Platelet-based attempts to distribute growth factors to wounds have been established and have demonstrated to be effective in chronic wound healing.<sup>13,14</sup>

PRP therapy is a platelet concentrate containing at least five growth factors that aid wound healing. PRP may also aid in the fight against infections by attracting white blood cells and releasing bactericidal substances from platelets. Although much of the research suggests that PRP can help with healing, a few trials have come up short. Negative outcomes could be due to differences in equipment and study strategies.<sup>15</sup>

Mohammadi et al., 2016 offered an excellent review of the difficulties that lead to false-negative outcomes, emphasizing the importance of autologous PRP containing viable active platelets in adequate concentration to assist healing. When these requirements are met, the vast majority of studies show that using PRP improves healing significantly.<sup>16</sup>

Several studies have shown that using chitosan as a biologically active dressing can help with wound healing. The administration of chitosan to open wounds in dogs resulted in exudate, that has a high growth factor activity, as well as inflammatory cell infiltration and granulation tissue formation, as well as angiogenesis.<sup>17</sup>

In the current study the mixed PRP with chitosan was used in 95:5 ratio because this ratio has best homogeneity in mixing according to some tests including scanning electron microscope and FTIR EDS test. A closer inspection to the SEM images indicates that chitosan possesses a nanoparticles size and distributed inside the PRP solution homogeneously.

Mohammadi et al, 2016 use the mixture of chitosan and PRP to see how it affects wound healing and observed that the mixture has beneficial effect in wound repair in animal and human being.<sup>18</sup>

In this study there were higher bone formation in the chitosan/PRP group in comparing with other group in 4<sup>th</sup> and 10<sup>th</sup> week

and this result disagree with<sup>9</sup> this may be due to mixture ratio which was in equal amount and this is affect the homogeneity and presence of obvious nano cracks which may lead to non-homogenous mixture<sup>18</sup> which were examined by SEM which may lead to a huge remnant of chitosan in the defect side and impair bone formation. Also may be due to the duaration of healing which are more in our study.

And the result agrees with Sequando et al., 2018 and Shwu et al., 2009 that used the mixture of chitosan and PRP with other CASO4 and showed more bone formation compared with chitosan alone.<sup>19,20</sup>

## CONCLUSION

The use of PRP and Chitosan together speeds up bone healing and regeneration

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