REPAIRING AN AUNILATERAL CRITICAL-SIZED MANDIBULAR DEFECT IN RABBITS USING HYDROXYAPATITE MATRIX, POLYLACTIC-POLYGLYCOLIC ACID (HA/PLGA) SCAFFOLD, AND PLATELET RICH PLASMA[PRP]

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ABSTRACT

Despite extensive research, the use of autologous platelet-rich plasma (PRP) to treat major bone abnormalities remains controversial. As a substitution, allogeneic PRP from donors who have been thoroughly vetted not just produces a more accurate and reliable treatment but also does not require drawing significant amounts of blood. Allogeneic PRP is nevertheless usually understudied, especially given its immunogenicity in this use. **This study's objective** was to assess the effects of using PLGA/HA scaffold together with platelet-rich plasma (PRP) on bone regeneration of critical-size mandibular defects in a rabbit model.

Material and methods: A total of 18 mandibular defects were made, and three groups (each n = 6) were created. The first group received a hydroxyapatite matrix and polylactic-polyglycolic acid HA/PLGA) scaffold in combination with PRP. The second group received only HA/PLGA scaffolds. The third group, which served as the control, had a critical-sized defect left empty. Specimens were collected and evaluated by means of micro-CT scans and histological analysis after 3 months.

Results: PRP + PLGA/HA led to more extensive new bone formation ($p < 0.05$) in contrast to the PLGA/HA scaffold only group and the control group.

In conclusion, In a mandibular rabbit model, a mixture of PRP, PLGA, and HA enhances bone repair. This research supports the use of autologous (PRP) as an over-the-counter treatment for bone repair.

KEYWORDS: platelet-rich plasma (PRP); synthetic scaffolds; bone regeneration; tissue engineering.

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INTRODUCTION

There is substantial morbidity and mortality associated with bone abnormalities [1].Trauma, tumor removal, congenital abnormalities, and degenerative illnesses are the leading causes of bone fractures [2]. Bone fracture consolidation is a time- and space-consuming biological process that involves a wide variety of cell types interacting with one another. Inflammation, ossification, and remodeling are only some of the processes that follow the formation of a blood clot, which occurs when the plasma coagulation cascade is activated [3]. Although bone tissue has a strong regenerating potential in general, this capacity is diminished in regions with significant expansions, delaying consolidation [4].

Despite mounting evidence that autologous bone grafts represent the gold standard of care, they are presently regarded therapeutically limiting due to their limited availability and the morbidity associated with the donor location [5, 6]. Therefore, tissue engineering using biotechnology entails introducing novel therapeutic techniques through the creation of composite biomaterials that may respond to the injury's surrounding environment to promote a speedy and risk-free healing. [7, 8]

The goal of creating a composite biomaterial is to combine the beneficial qualities of multiple materials into a single product with enhanced biological performance over that of any of the component parts used individually. Because of their optimal three-dimensional (3-D) shape for guiding cell adhesion and proliferation and their ability to carry or store nutrients, water, cytokines, and/or growth factors[8], these biomaterials are often built in the scaffold format.

Hydroxyapatite (HA) is a mineral made mostly of phosphate and calcium with bio compatibility and osteoconductivity qualities match the mineral structure of real bone, making it one of the most often utilized materials for this purpose. Although HA nanocomposite materials have been proven to enhance osteoblast growth and differentiation in vitro [9], they lack mechanical stability. This motivates the search for synthetic polymers suitable for this purpose.[10]

One of the most widely used synthetic biodegradable polymers, poly (lactic-co-glycolic acid) (PLGA) has a series of predictable and acceptable breakdown rates in comparison with tissue development rates, making it highly biocompatible. However, the quick decomposition of PLGA will produce acidic chemicals, leading to a low local pH and poor toughness. Increased cell activity and better bone formation may be attributed to the incorporation of ceramic materials.[11]

By modulating bone cell chemotaxis, proliferation, differentiation, and synthetic action, osteoinductive growth factors control physiological remodeling and fracture healing. Multiple growth factors have been shown to promote bone repair[12]. Some examples of these include insulin-like growth factors (IGFs), transforming growth factor b (TGF-b), and bone morphogenetic proteins (BMPs).

Platelet-rich plasma (PRP) is a natural source of several different types of growth factors, including IGF, platelet-derived growth factor (PDGF), PDGF, TGF-b1, TGF-b2, epidermal growth factor (EGF), and epithelial cell growth factor (ECGF) [13]. It eliminates the possibility of spreading illness since it is self-contained. Additionally, PRP may be conveniently extracted from autogenous whole blood in only two centrifugation stages on the day of surgery. The efficacy of PRP to promote bone and soft tissue healing has been supported by basic research[14].

MATERIAL AND METHODS

Animals

Sixteen New Zealand white rabbits, aged 6 months, weighing between 1.5 and 2.0 kg, were

utilized in this investigation; they were housed individually, fed a regular diet, and given free roam of the facility. The following 3 groups (each n = 6) were formed: the critical-size defect was filled with: (1) PLGA/HA scaffold and PRP; (2) PLGA/HA scaffold only (3) and control groups left an empty defect. All animals were treated humanely under the ethics approval committee of Minia University **(Approval 22/2/2021: [No. 486]**

In vitro study

Ceramic scaffolds and PRP

At 25 degrees Celsius for 24 hours, a 3:1 w/w mixture of HA and PLGA (50/50) solutions in 0.2% chloroform was created. After the HA/PLGA solution was mixed and dissolved, it was poured into a cylinder-shaped mold and frozen for two hours at -20 degrees Celsius before being lyophilized. The 18 wells were sized to accommodate tiny cylinders made from HA/PLGA scaffolds (9 mm in diameter and 2 mm in height) [15].

Loading and application of PRP in the ceramic scaffold

We used a protocol described by Li et al. [16] to create au-PRP. Blood clots were checked for by withdrawing 9 ml of peripheral vein whole blood into a sterilized centrifuge tube holding acid citrate dextrose solution and anticoagulants on the initial day of treatment. After being centrifuged in a frozen centrifuge at 600 rpm for 15 minutes, the red blood cell concentrate was discarded instantly. PRP was separated from platelet-poor plasma by centrifuging the residual plasma at 1,135 g for 7 minutes, as described above in the "preparation of PRP" section. We used a Sysmex XE-2100 (Nigale, Chengdu, China) automated blood cell analyzer to determine the platelet count. Prior to implantation, the ceramics in the PRP group were treated with 40 microliters of freshly prepared PRP.

In vivo study

Surgery

An intramuscular (I.M.) injection of ketamine (40 mg/kg; Alfamine1) and xylazine (5 mg/kg; Xylazinbio1) was used to put the animals to sleep. The surgical incision site was shaved, cleaned with povidone-iodine solution, and made sterile after the administration of general anesthesia. External incision was made in mandibular base of ~2cm by lancet as long as layer dissection, and sub periosteal flap was extended to the muscle layers till reaching the bone . In all animal models, critical-size flaws were drilled out with a hard drill #702 using a
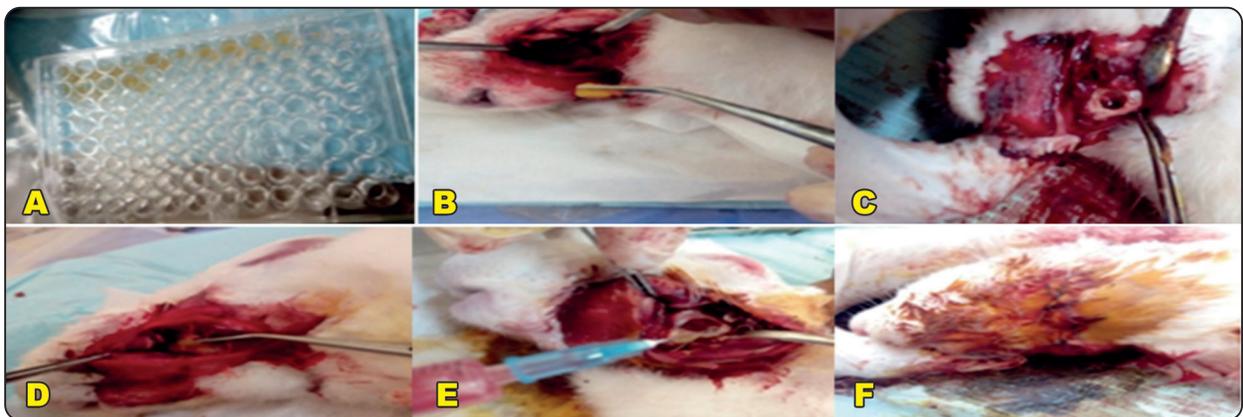

Fig. (1): Methods of surgical procedure: A: PLGA/HA before placement B: The mandibular periosteum Was elevated C: A circle bone segment (3-cm defect) was removed: D: Placement of scaffold in the surgical side E: Placement of scaffold HA/ PLGA/PRP in the surgical F: Suture by layers with silk 4-0

slow-speed hand piece and irrigated with sterile saline solution 0.9% on a continuous basis. The bone tissue was removed with a sharp excavator[17]. Finally, the flaps were repositioned carefully and sutured in layers.

Animals were euthanized using an overdose of anesthetic drugs (ketamine and xylazine) on the third postoperative month. The area containing the critical-sized bone defect was then surgically excised and sent to the processing slides for further examination.

Radiographic evaluation

Micro-Computer Tomography

After the animal was sacrificed, the bone samples were removed and transported with a wet towel to the [the Ora-scan. Sohag]'s core for m-CT examination. Microcomputer tomography (m-CT) (Fan beam Micro-CT) was used to analyze the samples. The m-CT system's X-ray source micro focus produced a 7 mm diameter beam with a peak voltage of 36 kV. Water was used to fill a sample container before the specimens were added. The blocks were aligned such that their longest sides were parallel to the sample holder's longest side. A high-resolution protocol was used, with slices 120 mm thick, a 60 mm feed, and 60 mm pixels. Up to 180 slices were scanned perpendicular to the block, however this number varied with the length of the specimens. It was also possible to reliably differentiate between the scaffold and freshly created bone by determining the ranges and means of the gray levels typical of each. In order to ensure the validity of the discriminating criteria[18], -CT slices were compared with comparable histology sl ides.

Histological examination of samples

The histology lab received bone samples, which were then treated in 10% formaldehyde for 5 days. The samples were then decalcified using 10%

formic acid (diluted with distilled water; Sigma Chemical Co., St Louis, MO, USA) for 10 days at room temperature following the aforementioned protocol. Following this, standard tissue processing was performed to get the samples ready for light microscopic analysis. Hematoxylin and eosin (HE) staining was performed on six randomly chosen cross slices from each 6 mm thick sample [19]. Three successive fields were photographed using a light microscope (Leica Microsystems, Wetzlar, Germany) for the qualitative analysis. In a blind study, everything was analyzed by a seasoned pathologist.

Statistical analysis

The information above is shown as means± standard deviations. SPSS 15.0 (SPSS Inc., Chicago, IL, USA) was used to analyze the data. , when P values were <0.05 the differences were deemed to be significantand, and highly significant when P<0.01 and P<0.001.

RESULTS

In vivo study

Figure 2: Macroscopic and radiographic images of the implantation sites at 3 months: After the study period, the healing of the rabbit mandibular defect was observed macroscopically and assessed using a micro-computer tomography in both the treated and control groups**.**

A) In Group I, the flaws healed completely, the newly produced bone looked and felt like normal osseous tissues, and the interface among the scaffolds and the host bone was distinct. (B) The flaw may be found in group II. Three months after implantation, a considerable piece of the remaining scaffold was still visible at the defect site, despite the fact that it had been partly filled by callus bone. (C) At 3 months post-implant, the control defects' soft consistency was a result of the lack of ossification and the presence of fibrous structures.

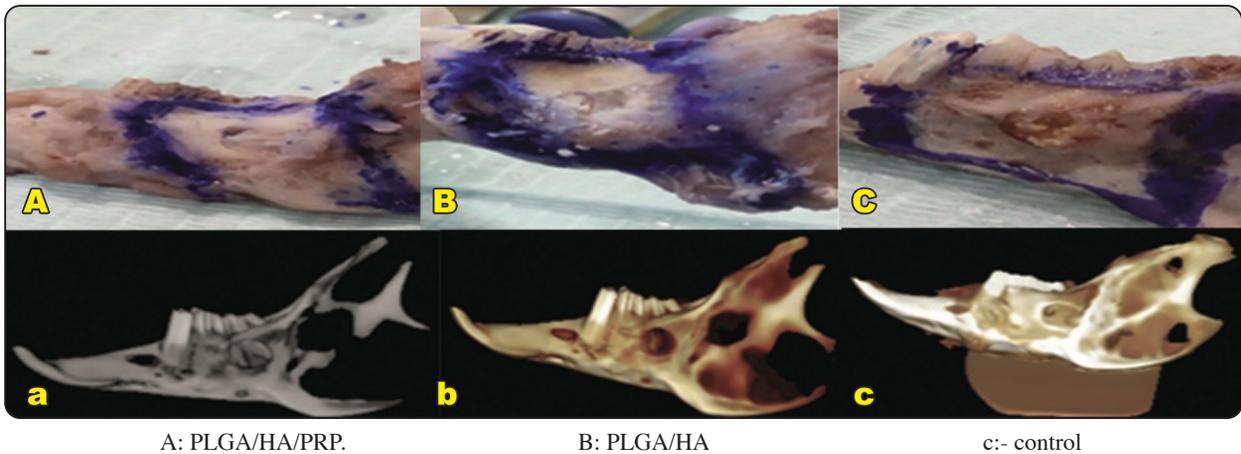A: PLGA/HA/PRP.   B: PLGA/HA   c:- control

Fig. (2)

B) (a) In the groups I the defects showed new bone formation within the defects with disintegration of the scaffold and evidence of reduced radiolucency (b) Radiolucent defect filling was seen in group II, suggesting less competent matrix production for the newly produced bone.. (c) The control defects presented a radiolucent circle inside defect, thus, the crucial bone defect model of the lesion was confirmed.

Statistics

Area Percentage of new bone formation and bone marrow in the critical sized of mandibular defects in the experimental groups: After completing every test, the mean as well as the average and standard deviation for each group was calculated. The Kolmogorov-Smirnov test and the Shapiro-Wilk test were carried out in order to determine whether or not the data adhered to a parametric distribution (normal distribution). We used a one-way analysis of variance (ANOVA) followed by a Tukey post hoc test so that we could contrast more than two groups in separate samples. The significance level was set at $P \le 0.05$. Statistical analysis was performed with IBM® SPSS® Statistics Version 20 for Windows

Statistical analysis of bone marrow percentages revealed a (p below 0.001) and (p below 0.001) difference between the PLGA/HA/PRP group and the PLGA/HA and control groups, respectively. Furthermore, PLGA/HA groups differed significantly from control groups where ($p<0.001$).

The proportion of new bone in the treated region was significantly higher in the PLGA/HA/PRP group compared to the PLGA/HA group and the control group, respectively ($p=0.006$) and ($p<0.001$). Furthermore, PLGA/HA groups differed with significance from control groups where ($p<0.001$).

Hematoxylin-eosin (HE) results:

Comparing the PLGA/HA/PRP group to the PLGA/HA and control groups, statistical analysis showed a ($p0.001$) and (p below 0.001) difference in bone marrow percentages. In addition, there was a statistically significant difference (p below 0.001) between the PLGA/HA and control groups.

There was a statistically significant increase in the formation of new bone in the treated region of the PLGA/HA/PRP group in comparison to the PLGA/HA group and the control group of people, respectively ($p = 0.006$ and p below 0.001, respectively). Furthermore, there was a statistically significant distinction (p less than 0.001) between the groups who received the PLGA/HA treatment and the control group.
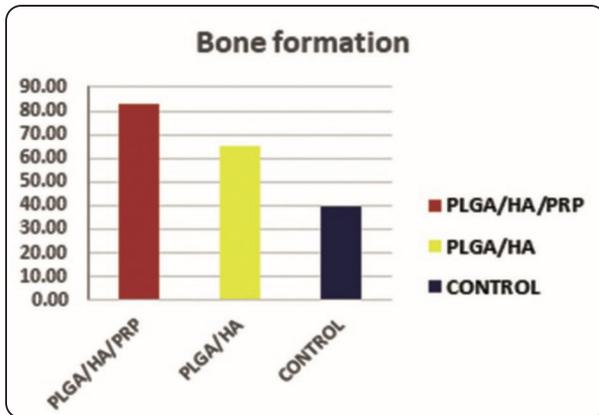
Fig. (3): Bar chart represents bone formation percentage between the experimental groups. There was a significantly higher (p < 0.05) bone formation between PLGA/HA/PRP and PLGA/HA group compared to the control group.
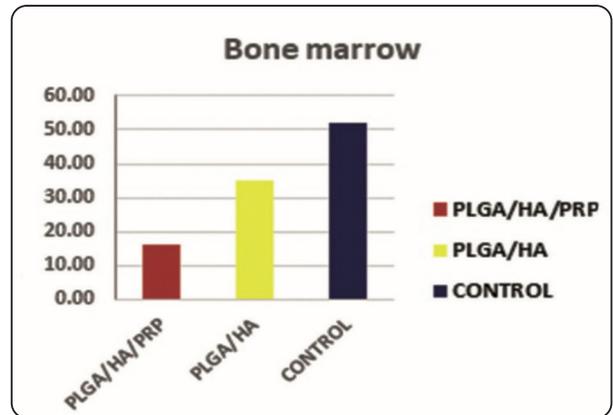

Fig. (4): Bar chart representing bone marrow percentage between the experimental groups. There was a significantly higher(p<0.05) bone formation between PLGA/HA/PRP and PLGA/HA group compared to the control group

Figure [5] [A-B-C-D] displays typical histological slices from 3 months post-surgery in the three experimental groups. After 3 months, the bone deficiency in the empty control group has been filled mostly by connective tissue. and only fibrous scar tissue has developed. In addition, the PLGA/HA only group showed a substantial amount of blood clot in the central portion of the defect, as well as the presence of small foci of connective tissue where it was also attainable to see the a greater amount of immature trabecular bone at the margins of defects;

after 3 months of healing, the grafting material was resorbed and replaced by mild new bone.

Due to the use of allogeneic platelet-rich plasma in the PLGA/HA/PRP groups from the start, two rabbits died on the first day and one rabbit died on the second day following surgery (al-PRP). However, when platelet-rich plasma (PRP) was generated using the patient's own platelets (au-PRP), a common form of PRP, there was no evidence of infection at the site of the surgery in the rabbits, and the remaining rabbits survived to
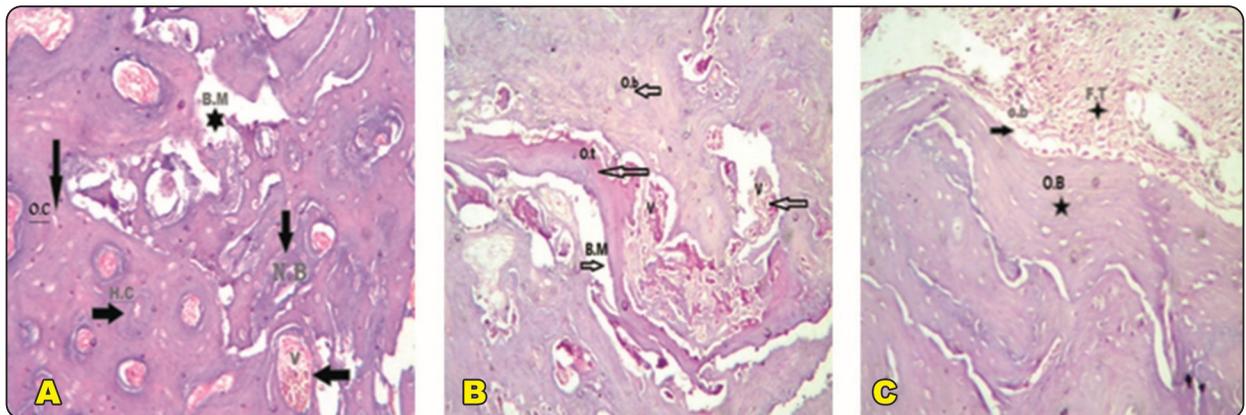

Fig. (5) Photomicrographs of decalcified section in the critical sized of mandibular bone defect in the three experimental groups after 3 months of implantation. (A) PLGA/HA/PRP group showing newly formed bone composed of osteon , Haversian canal [H.c] , osteocyte [O.S] a (B) PLGA/HA scaffold only group showing newly formed osteoid tissue in the central region of the defect with wide bone marrow spaces[B.M]and new vascularization [v] (C) Control group [empty bone defect showing old bone and osteoclast on the surface of the resorbing bone [o.c] and osteoblast on the surface of newly formed bone and fibrous tissue scar[F.t] nd. Coloration: hematoxylin and eosin (HE), bar = 40 μm, objective increase x10.

the end of the study. There were no indications of infection or negative surgical outcomes in any of these rabbits. According to histology, PLGA/HA/PRP showed that the scaffold material was resorbed and replaced by new bone that had advanced stages of remodelling and was completely filled with lamellar bone tissue with abundant osteocytes.

DISCUSSION

Bone defect models are the gold standard for studying the effect of stimulatory agents and biomaterials on healing [20]. In this investigation, researchers used a model in which a rabbit's mandible had a critical-sized lesion on only one side. The notion that platelet-rich plasma (PRP) paired with a hydroxyapatite matrix and a polylactic-polyglycolic acid (HA/PLGA) scaffold enhances bone regeneration in a critical-size mandibular bone defect was put to the test in the current study. Data from m-CT and histological analyses performed 3 months after implantation suggest that PRP dramatically improved bone regeneration.

Both autologous and allogeneic platelet-rich plasma (au-PRP and al-PRP, respectively) are common types of PRP, depending on the donor's blood type. In both human and veterinary medicine, au-PRP has shown promise as a supplementary treatment for speeding wound healing in animals. [21, 22]. Autologous platelet-rich plasma (PRP) for the healing of large bone lesions has been the subject of much study, but its clinical use has been limited by conflicting results. Platelet-rich plasma (PRP) quality varies greatly depending on platelet count, growth factor concentration, and growth factor activity. [23] may help to explain the differences of opinion around its use. Possible contributors to this diversity include centrifuge settings, blood collection reagents, and platelet activation protocols[24].

However, allogeneic PRP for bone defect repair has received less attention because to concerns about its immunogenicity [25]. One possible means of "immunogenicity reduction" is that, since al-PRP is

a gel, it may be completely dissolved and absorbed in the topical wound, so that very little al-PRP enters the bloodstream and bypasses major alloantigens (human leukocyte antigens [HLA] and human platelet antigens [HPA]). The immunogenicity of platelet surface antigen may be diminished by platelet activation because of changes in both the structure and the levels of platelet surface antigen[26].

Because of its excellent mechanical characteristics, biocompatibility, and biodegradability, poly (lactic-co-glycolic acid) (PLGA) has been extensively employed for in vitro and in vivo bone regeneration investigations and has received FDA approval. Lactic acid and glycolic acid are two of its byproducts, and they may cause mild to severe local irritation, However, the production of acidic substances during the rapid degradation of PLGA will result in low-local pH and poor toughness. The addition of ceramic materials is beneficial for enhancing cell activity and promoting bone formation [27].

Bone regeneration and remodeling of treated defects were monitored by CT radiographs and histology methods. The results of radiographic assessment in this study indicated that the defects treated with PLGA/HA/PRP were well healed There were no major discrepancies between the newly created bone and typical osseous tissues in terms of hardness or physical appearance, and the interface between the scaffolds and the host bone was distinct. The defect was partly filled by callus bone in the scaffold-only group, although a considerable amount of residual scaffold was still visible in the defect region.

On the other hand, the control defects presented few newly formed bone marrow spaces. **Wiltfang et al.,** [28] came to a similar conclusion, reporting that there was minimal improvement in bone regeneration after treatment of a critical-sized bone lesion with bone replacement materials..

Histological evidence further supported the micro-CT findings. In our study, Bone fracture in the location that got the combination of PLGA/HA/

PRP scaffold exhibited stronger healing signals, reflecting the radiological repair due to the radio density rising scale, as determined by histology investigation three months after transplantation. The percentage of residual connective tissue was lower in this group and the percentage of bone tissue was higher than in the group treated with a scaffold alone. Tomographic investigations corroborated the microscopic findings, showing an increase in bone tissue in the PLGA/HA/PRP-treated specimens.

Similar results were shown in a prior research when PRP was given to bone grafts for the restoration of mandibular abnormalities **by Marx et al.,** [29] radiographic maturation occurred more quickly and bone density was higher than with bone grafts alone.

An increased concentration of released growth factors is the primary idea behind using platelet-rich plasma (PRP) in combination with bone transplants., which in turn will improve the initial bone-healing response, as reported by **Jakse et al**., [30]. Eventually, PRP's direct effect will diminish, but the faster rate of bone regeneration caused by the physiological processes will persist

However, a less developed bone formed in the groups who got just the PLGA/HA scaffold. In comparison, the control group with no implants showed no new bone development and several voids that had not yet healed. The results agree with those of prior studies. According to **Zhang et al**., [31]. HA's conductive action has made it a popular component of bone scaffolds, where it is employed both alone and in combination with other substances. By expanding the calcium surface for osteoblast ossification, modified HA particles may help stabilize the mechanical characteristics of PLGA scaffolds, enhancing conduction ability.

CONCLUSIONS

Our results indicate that autologous platelet-rich plasma (PRP) could pave the path for an innovative BTE treatment method for the treatment of massive bone defects by causing neovascularization and speeding up the development and maturation of new bone with little effect on the immune system. When au-PRP is scarce, the ultimate usefulness of al-PRP has to be investigated further, and this requires a large-scale study.

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