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Assessment of Biphasic Calcium Phosphate mixed with injectable platelet rich fibrin (i-PRF) on healing of surgically created bone defects in a sheep animal model (A Histological Analysis)

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Abstract

Aims: Assessment of Biphasic Calcium Phosphate mixed with injectable platelet rich fibrin (i-PRF) on healing of surgically created bone defects in a sheep animal model. **Materials and Methods:** In each tibia / radius of five sheep, three defects each measuring 7mm in diameter and 4mm in depth were created. The defects were filled with study materials and in the following order: from a proximal to distal orientation; first defect was filled with biphasic calcium phosphate alone, second left empty to be filled by physiological clot and the third with i-PRF mixed with biphasic calcium phosphate. Histological examination of bone defects was made to assess bone formation at fourtime intervals (two, four, six and eight weeks) post-surgically. **Results:** Regarding bone formation, histological findings showed the presence of a significant difference within the time intervals in the BCP+i-PRF group and in the BCP group when compared with control group with the highest mean being at eight weeks post-surgery in the BCP+i-PRF group. **Conclusions:** Both BCP and i-PRF, enhanced bone formation when compared to the control group and throughout the period of study and as disclosed by histological findings.

الخلاصة

الأهداف: تهدف الدراسة الى تقييم كثافة العظام مع فوسفات الكالسيوم ثنائي الطور وحدها أو مختلطة مع الفيبرين الغني بالصفائح الدموية القابل للحقن في عيوب العظام التي تم إنشاؤها جراحيًا في عظم الساق للأغنام عن طريق التقييم النسيجي. **المواد وطرائق العمل:** بقطر 7ملم وعمق 4ملم تم إجراء ثلاثة عيوب قياسية في العظم تم ملء العيوب بالترتيب التالي: العيب الأول ملى بفوسفات الكالسيوم ثنائي الطور وحده ، والثاني ترك فارغًا ليتم ملؤه بخثرة دموية والثالث ملى بالفيبرين الغني بالصفيحات الدموية القابل للحقن ممزوجا بفوسفات الكالسيوم ثنائي الطور تم استخدام التقييم النسيجي لغرض تقييم كثافة العظام خلال الفترات الزمنية (اسبوعان , أربعة أسابيع , ستة أسابيع و ثمانية أسابيع) . **النتائج:** أظهرت النتائج وجود فرق كبير ذو دلالة إحصائية وخلال الفترات الزمنية التي تم الدراسة عليها في مجموعة الفيبرين الغني بالصفيحات الدموية القابل للحقن ممزوجا بفوسفات الكالسيوم ثنائي الطور وفي مجموعة فوسفات الكالسيوم ثنائي الطور وحده عند مقارنتها مع المجموعة الضابطة ، وأيضًا عند المقارنة بين المجموعات تم الكشف عن اختلاف كبير ذو دلالة احصائية ، مع أعلى متوسط هو ثمانية أسابيع بعد الجراحة لمجموعة الفيبرين الغني بالصفيحات الدموية القابل للحقن ممزوجا بفوسفات الكالسيوم ثنائي الطور. **الاستنتاجات:** قام كل من فوسفات الكالسيوم ثنائي الطور و الفيبرين الغني بالصفيحات الدموية القابل للحقن بتحسين كثافة العظام والحفاظ عليها طوال فترة الدراسة وكما تم الكشف عنها خلال نتائج الفحص النسيجي.

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INTRODUCTION

Bone is a living tissue composed of live cells set in a hard or firm framework [1], that constantly rebuilds its structure[2]. If a bone fracture occurs, it heals either directly or indirectly (most usually) with callus formation, which is a mixture of endochondral and intramembranous ossification, as opposed to primary healing, which occurs without callus development[3,4]. When trauma occurs, the continuity and vascular supply of bone is disrupted. The soft tissue envelope including the periosteum and surrounding muscles is torn, and numerous blood vessels crossing the fracture line are ruptured resulting in a lack of local oxygen and nutrients[5,6,7,8,9,10,11]. In all surgical professions, promoting healing is an ongoing concern. The development of surgical adjuvants for local healing stimulation is a popular focus of research in biomaterials and pharmaceutical sciences, with blood derived products being one of them.[12,13,14]. To date, many different materials can be found to fill bone defects. These can be allogenic bone, xenogenic bone, or bone substitutes which are defined as "synthetic, inorganic or biologically organic combinations which can be inserted for the treatment of a bone defect instead of autogenous or allogenous bone[15]. Among those various synthetic bone substitutes, biphasic calcium phosphate (BCP) have been widely used because of their chemical and structural similarity to human bone and its composition of less soluble hydroxyapatite (HA) to provide space maintenance and more biodegradable β-tricalcium phosphate (β-TCP) to control resorption rate[16]. Injectable PRF has been studied histologically and showed leucocytes (mainly lymphocyte) and platelet conglomerate, distributed uniformly throughout the analyzed specimen[17], unlike in PRF clot, where the cells are distributed non uniformly[18]. It was also discovered that the three-dimensional fibrin created in injectable PRF, together with growth factors, provide a controlled release system that maintains correct bioactivity throughout the healing process [19]. In this study we used BCP with **i**-PRF together to see their effect on bone healing.

Key Words: β-tricalcium phosphate, biphasic calcium phosphate, injectable platelet rich fibrin.

MATERIALS AND METHODS

Approval of study was from the Scientific Research Committee / Department of Oral and Maxillofacial Surgery / College of Dentistry / Mosul University. Five male sheep of local breed, aged 1.5 to 2 years, weighing 40-45 kg (mean= 42.5kg) were included in the study. The animals were acclimated for two weeks before surgery and their overall health was evaluated to ensure there were no general or infectious diseases. Each sheep model was divided into four observation subgroups, with each of the five animals undergoing surgery. The tibias and radiuses of each sheep were

operated on at random intervals of two weeks between surgeries.

Preparation of i-Platelet Rich Fibrin:

The jugular vein was used to collect blood in sheep. Two – 10 ml blood samples were collected from each sheep and promptly centrifuged in plastic tubes without any coatings. The centrifuge cycle for preparing an injectable PRF is 700 rpm for 3 minutes, according to the preparation technique[20]. At completion of cycle, the tubes are removed out of the centrifuge, the blood has been divided into two parts. Red blood cells make up the bottom layer, while plasma, platelets, and coagulating components in their uncoagulated state make up the top layer. The separated plasma and platelets form a light-yellow colored layer in liquid form. The top layer is aspirated using a syringe. This is done by placing the tip of the syringe just above the junction of the 2 layers and carefully aspirating the top layer. The aspirate is a partially active injectable form of PRF. Once the injectable PRF is obtained, it can be mixed with any particulate bone graft.

Surgical procedure:

Surgery was carried out under sterile conditions and under general anesthesia. An intramuscular injection of a mixture of (10mg/ml/kg) Ketamine hydrochloride general anesthetic agent (Hameln / Germany) and (2mg/ml/kg) Xylazine sedative analgesic solution (Intercheme / Holland) was used for general anesthesia (induction and maintenance). With a no.15-scalpel blade, any residual fine fleece at the operation site was carefully scraped off after the animal was anesthetized. The surgical region was disinfected with a 10% povidone iodine (Iraq) solution. Local anesthetic with epinephrine 1:80,000 (New Static / Colombia) was infiltrated at the operative site for hemostasis. A 5cm long longitudinal incision was created in the skin and periosteum along the lateral surface of the tibia/radius bone. A trephine bur (Dentium Implant Systems / South Korea) with a 7 mm width and 4 mm depth level set on a straight angle handpiece (speed of 1000 rotations per minute) was used to create three standardized monocortical bone defects. During the preparation of the defects, the trephine bur was positioned perpendicular to the long axis of the bone surface. Three conventional bone defects of 7 mm width and 4 mm depth, about 6mm apart, were created in each tibia or radius under copious irrigation with cooled 0.9 % normal saline (Haidylena / Egypt). From proximal to distal, the defects were filled in the following order: first with biphasic calcium phosphate alone, second is left empty to be filled with physiological clot , and third with i-PRF mixed with biphasic calcium phosphate. For standardization, a pre-weighed amount of BCP (using an electronic weight scale (A&D GX-200)) was placed into the allocated defects using a small head sized spoon excavator. The flap is closed using 3.0 black silk suture. Intramuscular injection of antibiotic

Oxytetracycline 20 mg/ml / 10 kg B.W (Alamycin 10 / Norbrook / UK) was given immediately after surgery. The animals were kept in the animal house for the first week and then were free to eat and drink and checked by a veterinarian on a regular basis. Until the sutures were removed (at the tenth post-operative day), the bandage covering the incision was changed every three days and the wound was monitored for any signs of infection.

Histological examination:

Histological examination was performed by 2 blinded examiners. All harvested specimens were fixed in 10% buffered formalin (PH 7.3) for 2 weeks, then rinsed with water. After fixation was completed, the tibias/radius that included artificial bone defects were cut using automated a minimicrotom (Struers minitom, Denmark). Decalcification was performed by immersion of the bony specimen in 50% formic acid and 20% sodium citrate solution. The solution was changed on an alternate day basis for 6 weeks. All samples were retransferred into formalin for 48 hours before final preparation for sectioning. Dehydration of samples was achieved in ascending series of ascending ethanol concentration from 70%, 80%, 95%, and absolute alcohol and then were placed into xylol to substitute alcohol. In the infiltration step, samples were cut in 5µm thickness in serial sections with microtome, and hematoxyline and eosin staining was performed for microscopic examination. Then they were submitted to histopathological examination under light microscope (Light microscope/Optica / Italy). A histological evaluation of the bone defects at four-time intervals (two, four, six and eight weeks) in each bone defect were performed following the completion of the surgical operations and as a foundation for comparing the three groups.

Statistical analysis:

All histological scoring variables to be assessed were considered non-parametric and hence the following tests were used: Kruskal – Wallace test: to show the significance between each interval at 2,4,6 and 8 weeks in the same group. The Mann-Whitney U test was used to show significance between groups for histological analysis at the scheduled intervals. Significance was set at $p \leq 0.05$.

RESULTS

Healing was uneventful in all animals and no complications were observed up to the day of sacrifice. A total of 60 sample were evaluated. Table (2) demonstrating the mean rank of histological scores of each group at four time intervals, when comparing within groups, in the control group, Kruskal-Wallis test results of histological findings showed a significant difference between time intervals with identical scores (0) in the second and fourth week yet score (1) in the sixth week and score (2) in the eighth week as shown in Figure (1). In the BCP alone group,

Kruskal-Wallis test results of histological findings showed a significant difference between time intervals with scores (1) in the second week , score (2) in the fourth, sixth and eighth week as shown in Figure (2). In the BCP + i-PRF group, Kruskal-Wallis test results of histological findings showed a significant difference between time intervals with scores (1) in the second week, score (2) in the fourth and score (3) in the sixth and eight weeks as shown in Figure (3). Mann-Whitney test results for histological score assessment showed a statistical significance among the three groups with healing scores higher in the BCP + i-PRF group and BCP alone group respectively when compared with the control defect group ad at each time intervals shown in table (3).

Table (1) : Histological criteria for assessment of bone formation[24].

SCORE	PARAMETER	CRITERIA
0	Newly formed vessels	None
	Numbers of fibroblast	None to very minimal
	Osteoid (bone matrix)	None
	Bone	None
1	Newly formed vessels	Few blood vessels
	Numbers of fibroblast	Few fibroblast
	Osteoid (bone matrix)	Evidence of matrix osteoid
	Bone	Evidence of bone formation
2	Newly formed vessels	Moderate blood vessels formation
	Numbers of fibroblast	Predominantly fibroblast
	Osteoid (bone matrix)	Moderate bone matrix deposited
	Bone	Moderate bone cells
3	Newly formed vessels	Extensive blood vessels
	Numbers of fibroblast	Fewer number of fibroblast
	Osteoid (bone matrix)	Dense highly organized bone matrix
	Bone	Extensive bone cells

Table (2): Comparisons of histological scores of each group at four-time intervals. Values are Mean.

Groups	Two weeks interval	Four weeks interval	Six weeks interval	Eight weeks interval
Control	5.5	5.5	15.5	15.5
BCP	3	8	15.5	15.5
BCP+i-PRF	3	10.5	10.5	18

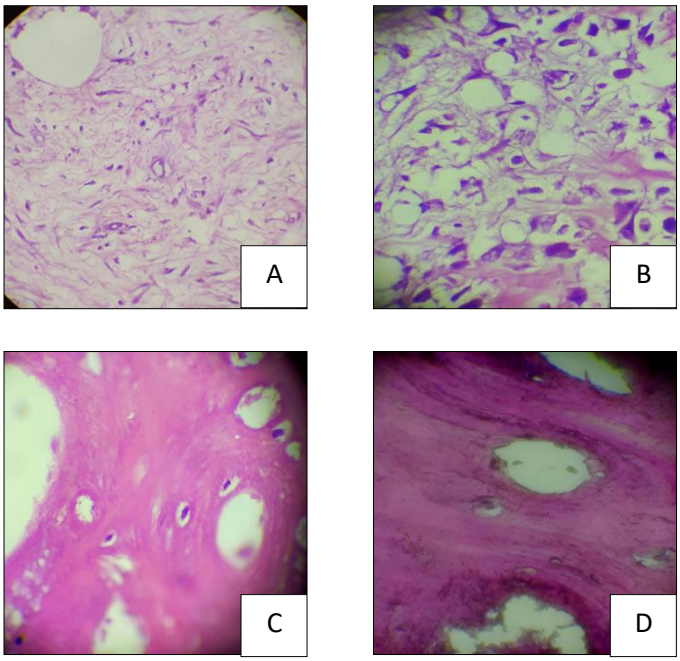

Figure (1) : Microphotographs of control groups at (A) two weeks with score (0), (B) four weeks with score (0), (C) six weeks with score (1), (D) eight weeks with score (2).

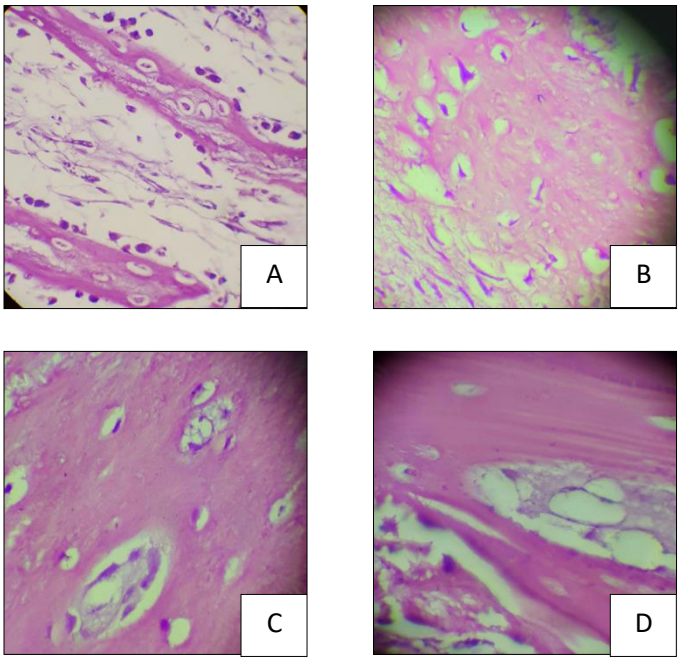

Figure (2) : Microphotographs of BCP groups at (A) two weeks with score (1), (B) four weeks with score (2), (C) six weeks with score (2), (D) eight weeks with score (2).

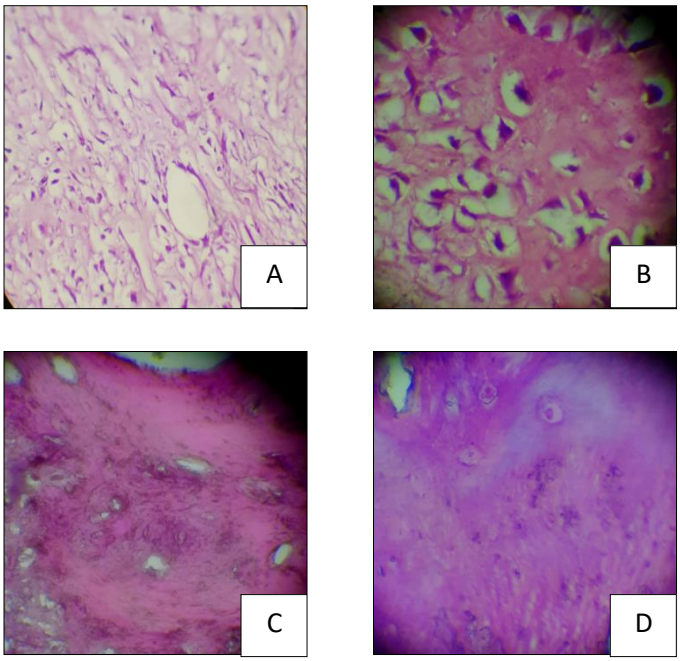

Figure (3): Microphotographs of BCP+ **i**-PRF groups at (A) two weeks with score (1), (B) four weeks with score (2), (C) six weeks with score (3), (D) eight weeks with score (3).

Table (3): Mann Whitney test comparisons between the three groups at each time intervals post-surgery. *Significance set as P≤0.05.

Groups	Two weeks interval	Four weeks interval	Six weeks interval	Eight weeks intervals
Control verse BCP	0.003*	0.005*	0.003*	0.003*
Control verse i-PRF+BCP	0.003*	0.005*	0.003*	0.003*
BCP verse i-PRF+BCP	1.000	1.000	0.003*	0.003*

DISCUSSION

Over the last 30 years, a variety of synthetic biomaterials have been developed as bone graft alternatives with the goal of overcoming the issues of autograft and allograft transplantation [21]. An ideal bone substitute material may act as a substrate for the migration, proliferation, and differentiation of cells invading from surrounding tissues, which would then be followed by bone tissue ingrowth[22]. In recent years, autologous platelet concentrates have acquired a lot of traction as a low-cost restorative treatment that can drive tissue neoangiogenesis [23]. Injectable platelet-rich fibrin (i-PRF) has been used as an autograft material to promote bone repair by releasing growth factors from the platelets[19]. Through histological examination, the current study assessed the accelerating effect of BCP on bone formation when associated with i-PRF. There are currently no experimental data in the literature on the combination of i-PRF and BCP that we are aware of. The current study's findings revealed that BCP has a good effect on bone regeneration at

all-time intervals studied. Histological findings showed an increase in bone formation which may be due to the increase in newly formed blood vessels , number of fibroblasts and osteoid tissue formation in all BCP+i-PRF groups and this finding came into agreement with the study by Bölükba et al. whom evaluated the efficacy of PRF mixed with biphasic calcium phosphate (BCP) on bone regeneration in surgically created bone defects in both tibias of 6 sheep. The defects were left empty or grafted with BCP, PRF, or BCP with PRF. Animals were sacrificed at 10, 20, and 40 days. The specimens underwent histologic and histomorphometric analysis and the results showed that there was histomorphometric increase in bone formation with the addition of PRF to BCP [16]. In addition, Liu et al., showed that the percentages of new bone formation and soft-tissue area were higher in the PRF group but were not significantly different [24].

CONCLUSIONS

Within the criteria of the current investigation, and at the conclusion of the experiment, the study indicated that both BCP and i-PRF enhanced bone formation throughout the study period and as observed by histological examination and as such, PRF promises to be a well-accepted minimally invasive method with favorable clinical outcomes in its modern forms.

Declaration of interest

The authors declare that there are no conflicts of interest regarding the publication of this manuscript

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