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RESEARCH ARTICLE

The impact of bone substitute combined with blood cell progenerators on the healing of surgical bony defects

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Abstract

Five local breed male sheep have been used in the study. Four 7-mm diameter, 4-mm deep bone defects were intentionally drilled in each sheep's tibia/radius for experimentation. General anesthesia surgeries were spaced 2 to 4 weeks apart. The first distal defect was left untreated as a control, whilst the second defect was subjected to a treatment involving a combination of standard-platelet-rich fibrin (S-PRF) and osteon III (hydroxyapatite+ Beta tricalcium phosphate (HA/β-TCP)). The third defect was treated with Advanced-PRF (A-PRF) and osteon III, while the fourth defect was treated with concentrated growth factors (CGFs) and osteon III. Two weeks following the final surgery, each sheep was sacrificed. Immunohistochemical analyses were performed. Immunohistochemistry revealed a statistically significant reaction to Ki-67 and osteopontine in the CGFs, especially at the end of weeks six and eight while no significant reactions to CD31 and CD34 antibodies at all study intervals.

Keywords: Immune markers, Immunohistochemistry, CD31, ki-67, CD34, Osteopontine, Concentrated growth factors.

Introduction

Addressing bone abnormalities resulting from trauma, tumor growth, infections, or surgical interventions remains a significant challenge in the realm of clinical and medical research (Kim *et al*., 2020; Liu *et al*., 2020). Various bone graft materials have been developed and are utilized in periodontal and implant surgeries to repair bony abnormalities (Miron, 2023).

Platelet rich fibrin (PRF) provides a straightforward and cost-effective approach of acquiring a natural concentration of autologous growth factors while minimizing invasiveness. PRF, or Platelet-rich fibrin, is frequently employed to expedite the healing process of both soft and hard tissues. This makes PRF a useful instrument in several medical domains (Mohanty *et al*., 2021)

The advanced-PRF (A-PRF) is a new modification that involves collecting the same volume of blood and subjecting it to centrifugation at 1500 rpm for a duration of 14 minutes (Alshujaa *et al*., 2023; Ghanaati *et al*., 2014. Both techniques result in the tube containing three separate components after the centrifugation cycle: red blood cells at the bottom, a fibrin clot representing the PRF in the center, and acellular plasma at the top (Dohan *et al*., 2006; Tadic *et al*., 2023).

In 2006, Sacco identified the concentrated growth factor (CGF) as a compound derived from the patient's own platelets, including a high concentration of growth factors and blood cells (Assadi *et al*., 2023; Rodella *et al*., 2011). CGF is obtained from whole blood samples without the addition of any other components. This is accomplished by a straightforward and standardized process that utilizes a specialized centrifuge. Platelet-rich plasma (PRP) and plasma rich in growth factors (PRGF) have a lower viscosity compared to CGF, as CGF contains a higher amount of fibrin. It closely resembles PRF (Isobe *et al*., 2017; Masuki *et al*., 2016).

This study aimed to investigate the efficacy of PRF regimens and CGFs when used in conjunction with osteon III for enhancing the healing of bone defects.

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Materials and Methods

Ethical Approval

The Animal Ethical Committee at the Dohuk Research Center (DRC) of the College of Veterinary Medicine, specifically the Department of Surgery, affiliated with the University of Duhok, approved this work. [DR2020106CV] was assigned as the study's reference number. The procedures followed Duhok University's ethical standards for the treatment and welfare of experimental animals at the Veterinary Theater.

Animal Model

A total of five male sheep, all of which were obtained from the same farm, were chosen for the study. All animals displayed a condition of robust health, devoid of any systemic illnesses. The animals had a preoperative fasting period of 24 hours. A mixture of procaine penicillin and streptomycin sulphate was administered prior to the surgical operation, with dosages of 24 and 30 mg/kg, respectively. Each sheep underwent random procedures on both tibias and radiuses, with a time interval of two to four weeks between each surgery. This resulted in a total of four surgeries on each sheep, and the observation period lasted twelve weeks. The animals were killed two weeks after the last procedure.

Anesthesia

An intramuscular injection of atropine sulfate at a dose of 1 mg/25 kg body weight was administered 10 minutes before to the start of anesthesia. In order to reduce the production of body fluids, suppress the activation of the vagus nerve on the heart, and avoid bradycardia. Following that, the patient was put under general anesthesia and kept under it by receiving multiple intramuscular injections of 0.1 mg/kg xylazine and 8 mg/kg ketamine.

Preparation of Platelet-Rich Fibrin from Blood Samples

The following are the basics of the blood collection and preparation procedures:

- Three syringes were employed to obtain 10 mL of blood from each sheep, which was thereafter subjected to quick centrifugation. The recommended technique for preparation involves a centrifuge cycle of 3000 rpm for 10 minutes when preparing S-PRF, and a cycle of 1500 rpm for 14 minutes when preparing A-PRF.
- The blood tube containing CGF was subjected to centrifugation using the following procedure: The sequence consists of 30 seconds of increasing speed, followed by 2 minutes of maintaining a speed of 2700 rpm, then 4 minutes at 2400 rpm, another 4 minutes at 2700 rpm, and finally 3 minutes at 3000 rpm. This is followed by a 36-second decrease in speed and coming to a halt (Qiao & An, 2017).

The Surgical Protocol

All five lambs received surgical operations and were divided into four observation subgroups based on a predefined sacrifice schedule. In order to reduce the likelihood of muscle damage, a straight cut of at least 6 cm in length was performed on the skin and periosteum along the inner side of the tibia and radius bones. The method utilized a trephine bur with dimensions of 7 mm width and 4 mm depth. The bur was attached to a handpiece with a straight-angled design. The minimum spacing between the four flaws was always maintained at 5 mm. To produce a control group, the original defect was just filled with blood. The second defect was repaired using a mixture of S-PRF and osteon III, the third defect was repaired using a mixture of A-PRF and osteon III, and the fourth defect was repaired using a mixture of CGF (particularly Sacco's membrane) and osteon III. The flaws were systematically repaired, beginning from a far position and advancing towards a closer position. Afterward, the absorbable collagen membrane was employed to conceal the faults., as illustrated in Figure 1.

The wound was closed, and the subcutaneous tissues were approximated with 0/2 polyglycolic acid sutures and 0/3 BBS sutures after the procedure. Furthermore, the affected region was treated with an aerosolized antibiotic called oxytetracycline and then properly covered with a suitable wrapping.

Statistical Methods

Repeated measures ANOVA was used to analyze the significance of changes within each group over time. Furthermore, repeated-measures ANOVA and Bonferroni correction tests were used to evaluate the clinical parameter intensities among four groups at different periods. A *p-value* of less than 0.05 was used to determine a significant level. All calculations were carried out with the Statistical Package for the Social Sciences 24 (SPSS 24; IBM Corp; United States).

Image Capturing

Digital photographs of the practical part all taken by canon digital camera 750D (canon/Japan) with SIGMA macro lens.

Digital photographs of the sections were captured by the camera-slide microscope system (optika-italy) in the College of Dentistry-Department of Oral and Maxillofacial Surgery-University of Mosul. The images were managed and captured by a computer program provided by the system (ProView program) at 4x,10× ,40x and 400x magnification.

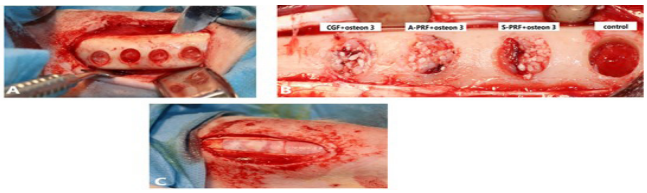

Figure 1: (A) Four defects with a 5 mm distance in between, (B) three defects filled with the mixture; the control one left empty.

Results

The process of healing had no evidence of any notable incidents in any of the animals. There were no apparent complications, and 20 samples were analyzed.

Immunohistochemistry

Four immune markers were used in the study, and their reactions were as follows:

Ki-67

No statistically significant variations were seen between the periods with the mean rankings of Ki 67 in the control (p = 0.612), standard (p = 0.557), and advanced (p = 0.377) groups. As shown in Table 1, the observed difference between the CGF and control groups was statistically significant (p = 0.007). However, there is an observed increase in the mean, median, and mean rank during week six, followed by a subsequent decline during weeks 8 and 12. Ki- 67 reactions are shown in Figure 2.

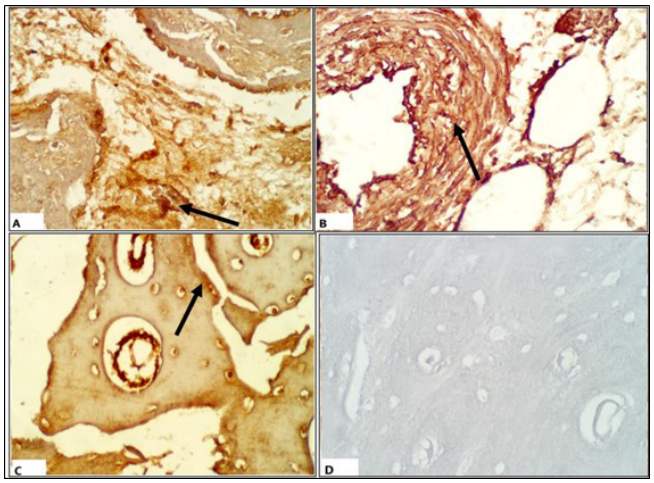

Figure 2: Ki- 67 reactions: (A) strong (++) reaction by the control group at week 2, (B) strong (++) positive of standard PRF +Osteon III at week 6, (C) strong (++) positive of the advanced PRF+Osteon III at week 8, (D) negative (-) reaction of the CGF +Osteon III at week 12. (400x)

CD31

No significant differences were detected in the CD 31 parameters between the different time periods in each of the following groups: control (p = 0.443), standard (p = 0.253), advanced (p = 0.455), and CGF (p = 0.368), as shown in Table 2.

CD34

No significant differences were detected between the different time periods in each of the study groups, as follows: control (p = 0.512), standard (p = 0.153), advanced (p = 0.271), and CGF (p = 0.057).

Osteopontin

In control (p = 0.503), standard (p = 0.468), and advanced (p = 0.157) groups, there were no significant differences in osteopontine parameters between the various periods. In the CGF group, the parameters increased during week 6 but declined during weeks 8 and 12 (p = 0.007). According

Table 1: Reaction to Ki 67 antibodies

Control	*Mean*	*(SD)*	*Median*	*Mean rank*	*P**	*Time periods*	*P***	*Time periods*	*P***
Week 2	2.0	(0.7)	2.0	2.4		2 X 6	NA	6 X 8	NA
Week 6	2.4	(0.5)	2.0	2.9	0.612	2 X 8	NA	6 X 12	NA
Week 8	2.2	(0.4)	2.0	2.7		2 X 12	NA	8 X 12	NA
Week 12	1.6	(1.1)	2.0	2.0					
Standard									
Week 2	2.0	(1.0)	2.0	2.2		2 X 6	NA	6 X 8	NA
Week 6	2.6	(0.5)	3.0	3.0	0.557	2 X 8	NA	6 X 12	NA
Week 8	2.4	(0.5)	2.0	2.7		2 X 12	NA	8 X 12	NA
Week 12	1.6	(1.3)	1.0	2.1					
Advanced									
Week 2	2.2	(0.8)	2.0	2.0		2 X 6	NA	6 X 8	NA
Week 6	3.0	(0.7)	3.0	2.9	0.377	2 X 8	NA	6 X 12	NA
Week 8	2.8	(1.1)	2.0	2.9		2 X 12	NA	8 X 12	NA
Week 12	2.4	(0.5)	2.0	2.2					
CGF									
Week 2	2.4	(0.5)	2.0	1.9		2 X 6	0.300	6 X 8	1.000
Week 6	3.6	(0.5)	4.0	3.5	0.007	2 X 8	0.518	6 X 12	0.042
Week 8	3.4	(0.5)	3.0	3.3		2 X 12	1.000	8 X 12	0.086
Week 12	1.8	(0.4)	2.0	1.3					

*By Friedman's Two-Way Analysis of Variance by Ranks. **Adjusted p value (Bonferroni post-hoc test).

Table 2: Reactions to CD 31 antibodies

Control	*Mean*	*(SD)*	*Median*	*Mean rank*	*P**	*Time periods*	*P***	*Time periods*	*P***
Week 2	2.0	(0.7)	2.0	2.0		2 X 6	NA	6 X 8	NA
Week 6	2.6	(0.5)	3.0	3.0	0.443	2 X 8	NA	6 X 12	NA
Week 8	2.4	(0.9)	3.0	2.8		2 X 12	NA	8 X 12	NA
Week 12	2.2	(0.4)	2.0	2.2					
Standard									
Week 2	2.4	(0.5)	2.0	2.1		2 X 6	NA	6 X 8	NA
Week 6	3.2	(0.4)	3.0	3.3	0.253	2 X 8	NA	6 X 12	NA
Week 8	2.8	(0.8)	3.0	2.6		2 X 12	NA	8 X 12	NA
Week 12	2.2	(0.8)	2.0	2.0					
Advanced									
Week 2	2.6	(0.5)	3.0	2.0		2 X 6	NA	6 X 8	NA
Week 6	3.2	(0.8)	3.0	3.0	0.455	2 X 8	NA	6 X 12	NA
Week 8	3.0	(0.7)	3.0	2.8		2 X 12	NA	8 X 12	NA
Week 12	2.4	(1.1)	2.0	2.2					
CGF									
Week 2	3.2	(0.4)	3.0	2.7		2 X 6	NA	6 X 8	NA
Week 6	3.4	(0.5)	3.0	3.1	0.368	2 X 8	NA	6 X 12	NA
Week 8	3.0	(0.7)	3.0	2.3		2 X 12	NA	8 X 12	NA
Week 12	2.6	(0.9)	2.0	1.9					

*By Friedman's Two-Way Analysis of Variance by Ranks. **Adjusted p value (Bonferroni post-hoc test). NA: Not applicable (as the difference was not significant between the groups).

Table 3: Reactions of samples to osteopontin antibodies

Intervals	*Mean*	*(SD)*	*Median*	*Mean rank*	*P**	*Periods*	*P***	*Periods*	*P***
Control									
Week 2	2.2	(0.8)	2.0	2.0		2 X 6	NA	6 X 8	NA
Week 6	2.8	(0.4)	3.0	3.0	0.503	2 X 8	NA	6 X 12	NA
Week 8	2.6	(0.5)	3.0	2.7		2 X 12	NA	8 X 12	NA
Week 12	2.4	(0.5)	2.0	2.3					
Standard									
Week 2	2.4	(0.5)	2.0	2.0		2 X 6	NA	6 X 8	NA
Week 6	2.8	(0.4)	3.0	2.8	0.468	2 X 8	NA	6 X 12	NA
Week 8	2.8	(0.4)	3.0	2.8		2 X 12	NA	8 X 12	NA
Week 12	2.6	(0.5)	3.0	2.4					
Advanced									
Week 2	2.6	(0.5)	3.0	2.4		2 X 6	NA	6 X 8	NA
Week 6	3.0	(0.7)	3.0	3.0	0.157	2 X 8	NA	6 X 12	NA
Week 8	3.0	(0.7)	3.0	3.0		2 X 12	NA	8 X 12	NA
Week 12	1.8	(0.8)	2.0	1.6					
CGF									
Week 2	2.6	(0.5)	3.0	2.3		2 X 6	0.850	6 X 8	1.000
Week 6	3.4	(0.5)	3.0	3.5	0.007	2 X 8	1.000	6 X 12	0.020
Week 8	3.2	(0.4)	3.0	3.1		2 X 12	0.850	8 X 12	0.086
Week 12	1.4	(0.9)	2.0	1.1					

By Friedman's Two-Way Analysis of Variance by Ranks. **Adjusted p-value (Bonferroni post-hoc *test). NA: Not applicable (as the difference between the groups was insignificant).

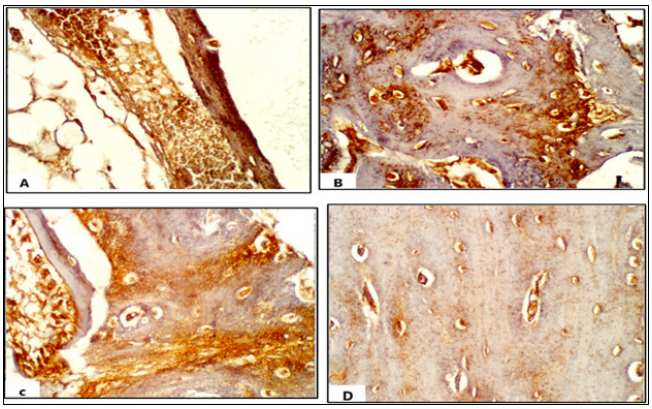

Figure 3: Reactions osteopontin antibodies, which appear as golden-brown patches (arrow). IHC Osteopontine. 400x. (A) showed (+++) very strong positive reaction of the control at week 2, (B) advanced PRF +Osteon III at week 6, (C) standard PRF+ Osteon III at eight weeks showed (+++) very strong positive reaction, (D) showed (+) positive reaction of the CGF +Osteon III group at the end week 12.

to Table 3, the difference between weeks 6 and 12 was statistically significant (p = 0.020) (Figure 3).

Discussion

CGF represents a significant advancement in fibrin matrix blocks, distinguished by a high concentration of growth factors. By employing various centrifugation speeds during the manufacturing of CGF, it is possible to get a fibrin matrix that is notably more compact and has a higher concentration of growth factors compared to other matrices (Rodella *et al*., 2011).

Concentrated Growth Factors

The unique centrifugation technique used in the production of CGF results in the creation of a fibrin network with improved qualities when compared to previous generations of blood-derived products. This improved network has increased cohesion, fibrin tensile strength, and stability. As a result, it is simple to manage, manipulate, and apply surgical defects. Furthermore, it gradually degrades over time, allowing for the continuous release of growth factors contained within the fibrin matrix. Bernardi *et al*.'s 2017 investigation validated these beneficial characteristics. (Bernardi *et al*., 2017)

This upgraded centrifugation approach also produces a matrix with superior physical properties and higher concentrations of growth agents, which accelerates bone synthesis and increases new bone creation (Öncü *et al*., 2016; Palermo *et al*., 2019).

Furthermore, because these concentrates are autologous and do not rely on bovine thrombin, any biocompatibility difficulties are avoided. According to Rodella *et al*.'s 2011 immunohistochemistry investigation, CGF indicates a significant fibrin matrix with great density and richness in growth factors. These characteristics promote the stimulation of cellular proliferation, matrix remodeling, and the formation of new blood vessels (neoangiogenesis). (Rodella *et al*., 2011)

The prospective impact of CGF on bone healing acceleration was studied immunohistopathologically in the current investigation.

Reaction to CD34 Antibody

CD34-positive cells were discovered in CGF aggregates by Rodella *et al*. (2011). The immunohistochemical staining findings in this research revealed the existence of CD34-positive cells that were evenly distributed across the fibrin network of a CGF membrane. Cells expressing the CD34 marker present in the circulatory system perform essential functions in the maintenance of blood vessels, as well as in the processes of forming new blood vessels (neovascularization) and the growth of blood vessels (angiogenesis). The observed architecture of the fibrin network implies the existence of CD34-positive cells, whereas the administration of CGF provides a diverse array of growth factors to these cells.

Although there was an increased presence of CD34-positive cells in the CGF group from the second week to the eighth week, this disparity did not reach statistical significance. The observed phenomenon can perhaps be ascribed to the structural arrangement of the CGF network, wherein the cells are confined. On the other hand, the results reported by Rodella *et al*. (2011) demonstrated a notable rise in the quantity of CD34-positive cells within the CGF group.

Given the limited availability of published data about the clinical significance of CD34-positive cells found in CGF, further investigation must be conducted in this area in future research endeavors (Rodella *et al*., 2011).

CD31 Reaction

CD31, alternatively referred to as platelet endothelial cell adhesion molecule 1, is a transmembrane protein expressed on the cells' surface. It has a molecular weight of around 130 kilodaltons. This entity belongs to the immunoglobulin superfamily (Newman *et al*., 1990). CD31 plays a crucial role in the mechanism of transendothelial leukocyte migration, facilitating cell-cell adhesion and facilitating antiapoptotic signaling (Fathi, 2023; Newman & Newman, 2003).

The expression of vascular endothelial growth factor, angiopoietin-1, fibroblast growth factor-2, and monocyte chemoattractant protein-1 was observed to be significantly present in CD31-positive cells. These angiogenic agents synergistically produce therapeutic neovascularization. (Yamashita *et al*., 2000)

Osteopontin

The Osteopontin (OPN) protein plays a pivotal role in promoting the attachment of bone cells to the bone matrix and initiates intracellular signaling pathways that impact the movement of osteoclasts within the bone tissue. This has

been observed in both *in-vivo* and *in-vitro* studies (Nielsen & Poellot, 2004). Using artificial Si scaffolds demonstrated osteoconductive, osteoproductive, and osteoinductive properties, leading to increased osteoblast proliferation and differentiation. In the CGF group, the study revealed an increase in these parameters in week 6 but a subsequent decrease at week 8 and week 12 ($p = 0.007$). Notably, there was a significant difference between weeks 6 and 12 ($p = 0.020$), although no significant difference was observed between the four groups at various time intervals.

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