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Potential Effect of a Novel Composite (Calcium Carbonate Nanoparticles/ Silver Nanoparticles/Advanced-Platelet Rich Fibrin) on Healing of Critical Bone Defect in Rabbits: Radiographic Evaluation

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ABSTRACT

The aim of the present study was to evaluate the efficacy of calcium carbonate nanoparticles (CCNPs)/Silver nanoparticles (Ag NPs)/Advanced-PRF composite to induce new bone formation and accelerate bone regeneration when be used to fill an experimentally critical defect in radial bone of rabbits. Twenty-four adult apparently healthy New Zealand white rabbits aged from 5-to 6 months and weighing 3.5 ± 0.5 kg were used in this study and were divided into two groups, group A: was left empty as a control. Group B: we used CCNPs/AgNPs/A-PRF composite. During observation, period rabbits were observed clinically and radiographically at 2,4,8,12 weeks post-operation. Results: the radiographic finding showed a great significant difference between the two groups and the CCNPs/AgNPs/A-PRF composite could make complete healing at the end of the observation period. Conclusion: radiographical studies revealed that CCNPs/AgNPs/A-PRF composite has exalted efficacy to induce new bone formation.

Keywords: AgNPs, A-PRF, Bone healing, CCNPs and Nano particles.

INTRODUCTION

The bone is a highly specialized tissue and can heal completely because its high regeneration capacity. However, defects formed due to trauma, infections, cysts and tumors decrease the regeneration ratio of the bone tissue, and can lead to problems in healing, so searching for bone graft substitutes become a critical need. These graft materials are used in order to facilitate and accelerate bone formation and to increase the physical endurance of that region (Gadallah, 1998; Lynch et al., 2008).

Bone substitutes act by one of three mechanisms: osteo-genesis, osteo-induction or osteo-conduction. The ideal bone substitute that offers these three is autologous bone, the ‘‘gold standard’’ for bone regeneration. However, the clinical application of autologous bone grafts is sometimes limited due to requiring an additional surgical harvesting site so searching for other alternatives is needed as allograft, xenograft or other biomaterials (Johansson et al., 2010).

In the past decades, studies on platelets revealed that platelets are responsible of releasing important biomolecules that is capable of stimulating the proliferation and activation of cells involved in healing process. These biomolecules include platelet-specific proteins and number of growth factors including transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF) and epidermal growth factor (EGF), and cytokines/chemokines and angiogenic factors (Choukroun et al., 2017). Dohan et al., (2006)

developed a simple method to prepare A-PRP without adding any anticoagulants or activators (thrombin or calcium chloride) called platelet rich fibrin (PRF) which considered as a new generation ofA-PRP. Sequentially, Ghanaati et al., (2014) improved PRF by decreasing speed and prolonging time of centrifugation, this led to a new protocol for PRF preparation called A-PRF. In vitro studies, A-PRF showed releasing of a significantly higher growth factors over time than PRF however, limited studies document its efficiency in vivo (Kobayashi et al., 2016).

CCNPs has received attention in bone regeneration due to its flexibility in preparation, tailor, biodegradation and osteo-conductivity and have a higher degradation rate compared with other synthetic bone substitutive ceramics, including nano-β-tricalcium phosphate and nano-hydroxyapatite, and it can enhance genes expression in specific osteogenic markers (Maleki, et al., 2019).

AgNPs had gained attention due to their exclusive biological, chemical, and physical properties in comparison to their large size equivalents. AgNPs was evidenced to be a most active material which possesses respectable antibacterial properties against dif-ferent microorganisms, for example, viruses, bacteria, fungi, and parasite (Sharma, et al., 2009). AgNPs promote the formation of the fibrous joint and the subsequent end joining of the fracture bone, induced and activated TGF-β/BMP signaling in the cells in the fracture zone (Zhang, et al., 2015).

The aim of the present study was to evaluate the efficacy of CCNPs/AgNPs/A-PRF as a new composite to induce new bone formation and accelerate bone regeneration.

MATERIALS AND METHODS

Animal model and housing:

A total of twenty-four healthy male New Zealand White rabbits aged (mean ±SD)5.0 ± 0.3 months and weighed 3.0 ± 0.5 kg were allowed to acclimatize to their new conditions for 2 weeks before the start of the study. All rabbits were free from systemic and musculoskeletal disorders. The rabbits were kept at a constant temperature of 23 ±1, 55% ±5% humidityand a 12 hours light / dark cycle.They had free access to standard diet and water adlibtum during the whole of experiment. Animals were managed according to the guide for care and use of laboratory animals approved by the Animal Care and Use Committee, University of Sadat City – faculty of Veterinary Medicine, Sadat City, Egypt.

Preparation of bone substitutes

The nanoparticles were synthesized in Nanomaterials synthesis and research unit, Animal Health Research Institute (AHRI), Agriculture Research Centre (ARC), Egypt.

Preparation of A-PRF membrane:

According to (Kobayashi et al., 2016) a 5 ml of whole blood obtained from central or lateral ear vein by using sterile syringe with needle 22G. The collected blood evacuated in sterile tube without adding anticoagulant.The blood sample centrifuged in a laboratory centrifuge (Unico; United Products & Instruments INC; USA) at 1500 rpm for 14 min at temperature 21 ℃. A fibrin clot was Formed at the middle of the tube between the red corpuscles at the bottom and acellular plasma at the top.The fibrin clot separated from the red corpuscles by using sterile scissors then compressed between two sterile gauze to obtain A-PRF membrane (fig. 1).

preparation of CCNPs powder:

According to (Hussein et al., 2020) a five g of stony coral powder was dissolved into 20 ml of 5M hydrochloric acid (HCl) 37%, (Emsure®; Merck KGaA, Darmstadt, Germany) to obtain calcium chloride ($CaCl_2$).The solution was filtered with filter papers. We used an electric mixer spun at 500 rpm for 1 h at 25℃ temperature to extract contaminated products from the solution. $CaCl_2$ was then diluted with 1 L double distilled water (DDW) to form stock solution. A Stock solution of K_2CO_3 was prepared by dissolving 5 g of K_2CO_3 ACS reagent, 99% (Sigma Aldrich, Steinheim, Germany) in 100 ml of DDW.10 ml of diluted $CaCl_2$ were then added to 50 ml of DDW followed by the addition of 10 ml of diluted K_2CO_3 at 1hr drop wise. The solutions were then left for 24 h to precipitate. The resultant product after removing of the supernatant solution was centrifuged at 6000 rpm for 15 min. The acidity of the resultant solution was then neutralized by washing three times with DDW. The resultant product was dried by using a hot air oven at 110 ℃ for 24 h.

Preparation of AgNPs:

Silver nitrate (AgNO3, 99% meets analytical specification of Ph. Eur., BP, USP,(Sigma-Aldrich) and tri-sodium citrate (C6H5O7Na3.2H2O, 99.99%) were used for

the preparation of silver nanoparticles. Silver colloid was prepared by chemical reduction according to (Van Dong et al., 2012). All solutions of reacting materials were prepared in deionized water. In typical experiment, 50 ml of 0.002 M AgNO3 was heated to boil. A 5 mL of 1 % trisodium citrate was added drop by drop. During the process, solutions were mixed vigorously and heated until change of color(grayish yellow) was noted. Then it was removed from the heating device and stirred until cooled to room temperature.

Preparation of CCNPs/AgNPS paste:

The technique was performed on the same basis mentioned by (Saraswathy et al., 2004), 1.5 g Gelatin was dissolved in 3 ml Hcl (N: 0.1) in water bath at 55 °C. The CCNPs powder and 5 ml of AgNPs were added to the previously prepared mixture and made into paste. The prepared pastes were packed in double wrapped plastic roll and sterilized by ultraviolet light for 30 second just before operation.

Characterization of prepared nanoparticles:

The particles size and surface charge of prepared NPs were characterized using a Malvern Zetasizer ZS-series (DLS, Malvern Co, UK). Thereafter, the morphological investigation of synthesized-NPs was detected by Transmission Electron Microscopy (TEM) (JEM-2100, JEOL). Phase identification and crystalline structure of CaCO3 NPs were performed by using X-Ray Diffractometer, XRD (SHIMADZU, XRD-6000) with Cu Ka radiation. The concentration of Ag NPs was quantitatively measured by ICP-MS instrument (Elan DRC-e, PerkinElmer, Germany).

Surgical approach and biomaterials implantation:

The right forelimb was clipped, shaved, washed and scrubbed with antiseptic (Betadine®). All animals were anesthetized by intramuscular administration of 40 mg/kg ketamine hydrochloride **(**KETALITE®: ELICE Pharma; Pakistan) and 5 mg/kg xylazine Hcl (Xylaject®: 2% sol. ADWIA Co., A.R.E). A 5-cm skin incision was made craniomedially over the forelimb. The radius was exposed by dissecting the surrounding muscles. A 14-mm critical-sized defect (Zhao et al., 2016) was then created by using surgical saw in the mid-diaphysis of the right radius. The defect of the animals in the group (A) was left empty. In the group (B) the bone defect was implanted with CCNPs/AgNPs paste and covered by A-PRFM (fig. 2) A prophylactic dose of antibiotic of Enrofloxacin (Curabiotic®: CureVet Co.; A.R.E) at dose 5 mg/kg body weight for three successive days, and stiches was removed after 7 days.

Radiographic evaluation:

The sequence of bone regeneration in terms of bone formation, cortical union and remodeling was monitoring by sequential radiographic images taken immediately post operation and at 2nd, 4th, 8th, 12th week of observation period by using X-rays apparatus (semens 300).The radiographic images were evaluated by two radiologists (without previos information), The radiographs were scored using Modified Lane–Sandhu scoring system (Parizi, et al., 2013) (Table 1).

Table (1): Modified Lane–Sandhu radiographic scoring standard by Parizi et al. (2013):

Item		score
Bone formation	No evidence of bone formation	0
	Bone formation occupying 25% of the defect	1
	Bone formation occupying 50% of the defect	2
	Bone formation occupying 75% of the defect	3
	Bone formation occupying 100% of the defect	4
Union (proximal and distal ends were evaluated separately)	No union	0
	Possible union	1
	Radiographic union	2
Remodeling	No evidence of remodeling	0
	Remodeling of medullary canal	1
	Full remodeling of cortex	2
Total points possible per category	Bone formation	4
	Proximal union	2
	Distal union	2

Remodeling	2
Maximum score	**10**

Statistical analysis:

All the results were reported as mean, median and standard deviations. A one-way ANOVA was used to carry out statistical analysis. $P \leq 0.05$ was considered as statistically significant. All the values were analyzed using the SPSS software (version 20.0; IBM, America).

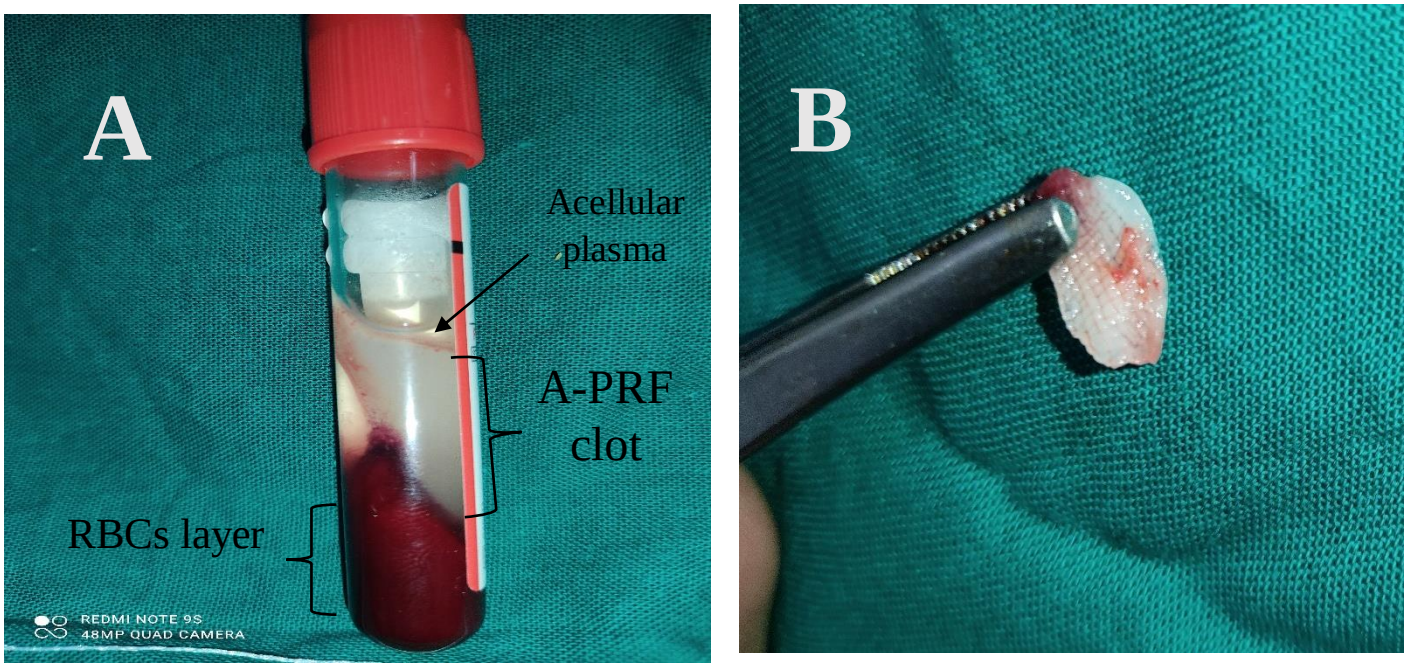

Fig. (1): Showing the A-PRF clot formed between the acellular plasma and the RBCs layer in the bottom (A), and A-PRFM after the A-PRF clot been compressed between two sterilized gauze (B).

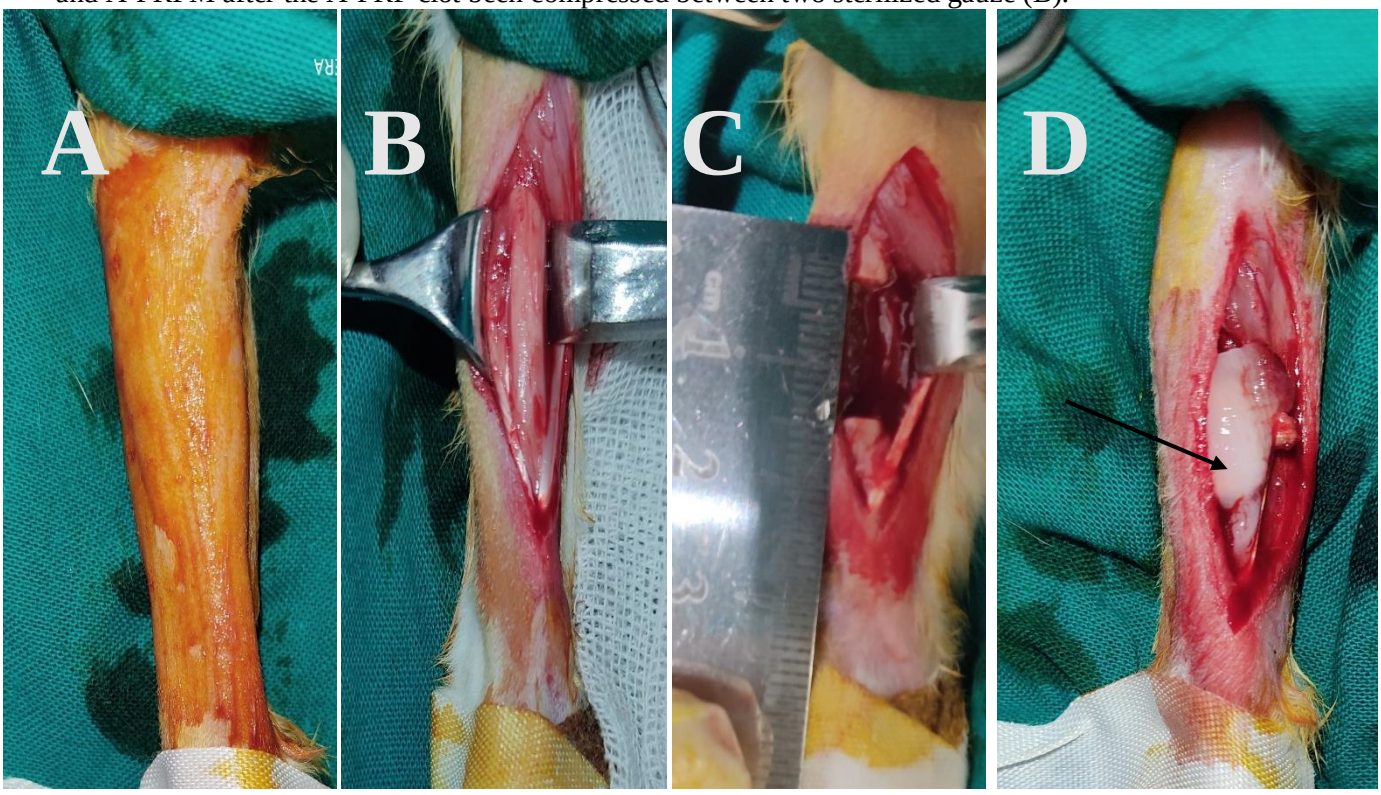

Fig. (2): A septic preparation of aneriomedial aspect of the right forelimb including clipping, shaving and washing with antiseptic solution (A). Incision in the aneriomedial aspect of the forelimb including the skin and muscles to expose the radius (B). A critical segmental defect with size 14 mm was made in the diaphysis of the radius (C). The created defect was implanted with CCNPs/AgNPs/A-PRFM composite (D) black arraw.

RESULTS

There were no infections or any complications observed in all operated cases along the observation period.

The radiographic imaging showed that adding bone graft substitutes to critical segmental radial defect with length 14 mm have great radiographic changes (fig, 8) comparing to control group (fig. 7). The statistical analysis showed significant differences in bone formation (table 2), proximal cortical union(table 3), remodeling(table 4) and total radiographic score (fig. 6).

Table (2): Showing bone formation score between groups A&B:

Groups	Times (weeks)					P-value
	zero	2w	4w	8w	12w	
Control	0 (0.0-0.0)^{c}	00.0-0.0)^{c*}	0 (0.0-1.0)^{c*}	1 (1.0-1.0)^{b*}	2 (2.0-2.0) ^{a*}	**0.000**
CCNPs/Ag NPs/APRF	0 (0.0-0.0)^{c}	0(0.0-1.0)^{c*}	2 (2.0-2.0)^{b**}	3 (2.0-3.0)^{b**}	4 (4.0-4.0)^{a**}	**0.000**
p. value	**-**	**0.195**	**0.003**	**0.004**	**0.000**	

Table (3): Showing proximal cortical union score between groups A&B:

Groups	Times (weeks)					P-value
	zero	2w	4w	8w	12w	
Control	0 (0.0-0.0)^{a}	0 (0.0-0.0)^{a*}	0 (0.0-0.0)^{a*}	0 (0.0-0.0)^{a*}	0 (0.0-1.0)^{a*}	**0.441**
CCNPs/Ag NPs/APRF	0 (0.0-0.0)^{c}	0 (0.0-1.0)^{c*}	1 (1.0-1.0)^{b**}	2 (1.0-2.0)^{a**}	2 (2.0-2.0)^{a**}	**0.000**
p-value	**-**	**0.195**	**0.003**	**0.001**	**0.004**	

Table (4): Showing distal cortical union score between groups A&B:

Groups	Times (weeks)					P-value
	zero	2w	4w	8w	12w	
Control	0 (0.0-0.0)^{c}	0(0.0-0.0)^{c*}	0 (0.0-1.0)^{c*}	1 (1.0-1.0)^{b*}	1 (1.0-1.0)^{a*}	**0.001**
CCNPs/Ag NPs/APRF	0 (0.0-0.0)^{c}	1(0.0-1.0)^{b**}	2 (1.0-2.0)^{a**}	2 (2.0-2.0)^{a**}	2 (2.0-2.0)^{a*}	**0.012**
p-value	**-**	**0.022**	**0.004**	**0.017**	**0.347**	

Table (5): Showing Remodeling score between groups A&B:

Groups	Times (weeks)					P-value
	zero	2w	4w	8w	12w	
Control	0 (0.0-0.0)^{a}	0 (0.0-0.0)^{a}	0 (0.0-0.0)^{a}	0 (0.0-0.0)^{a*}	0 (0.0-0.0)^{a*}	**-**
CCNPs/Ag NPs/APRF	0 (0.0-0.0)^{b}	0 (0.0-0.0)^{b}	0 (0.0-0.0)^{b}	1 (1.0-2.0)^{a**}	2 (2.0-2.0)^{a**}	**0.000**
p-value	**-**	**-**	**-**	**0.004**	**0.000**	

*, **: Medians and ranges with different asterisks superscripts in the same column are significantly different at P<0.05.

a,b,c,d: Medians and ranges with different small superscripts letters in the same row are significantly different at P<0.05.

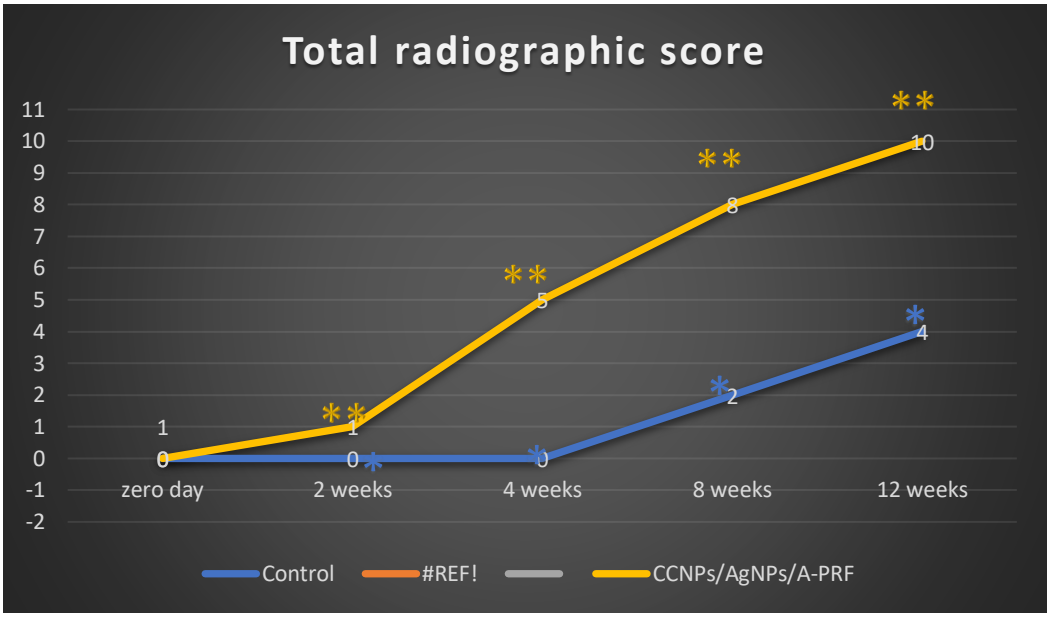

Fig. (3): The total radiographic score revealed significant difference between CCNPs/AgNPs/A-PRF group and control groups at the 2nd, 4th, 8th and 12th post-operative weeks.

*, **: Medians and ranges with different asterisks superscripts in the same time period are significantly different at P<0.05

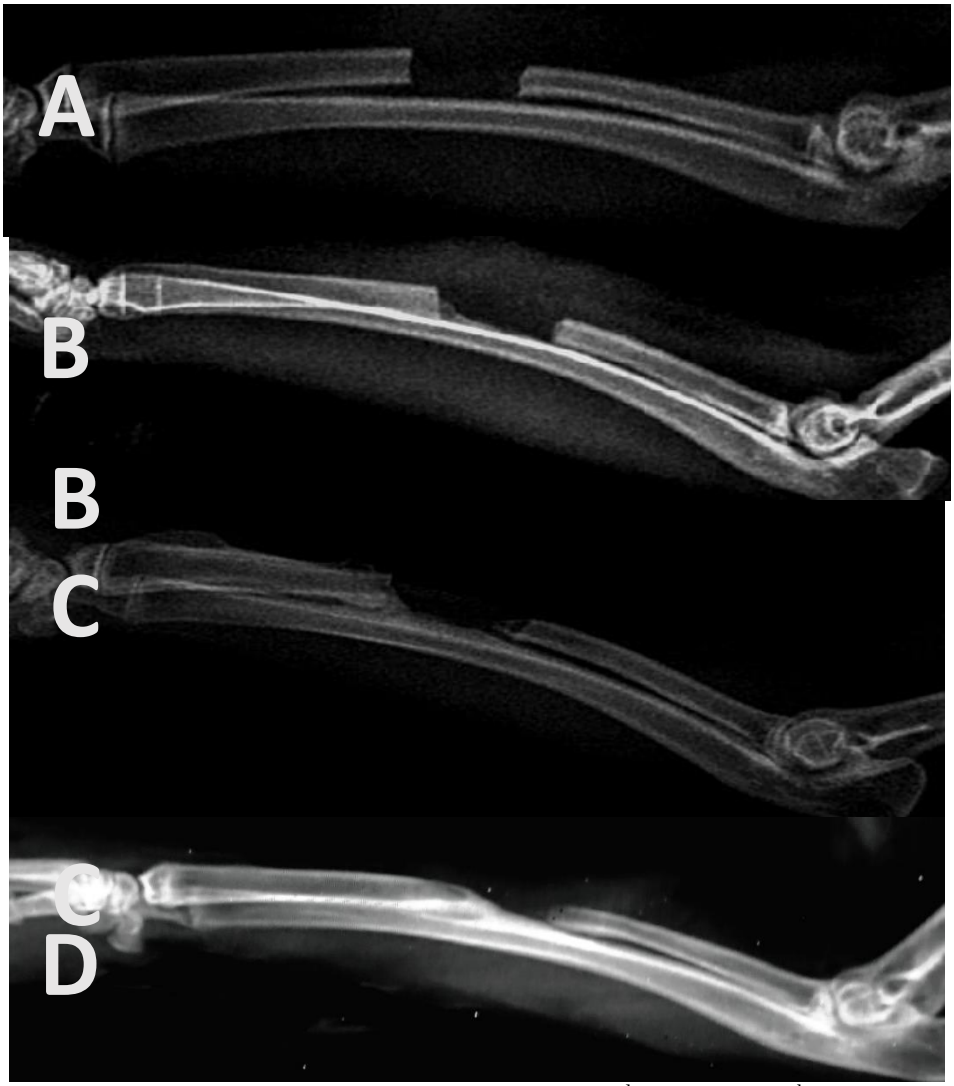

Fig. (4): Showing radiographic images of control group at 2nd week (A), 4th week (B), 8th week (C) and 12th week (D).

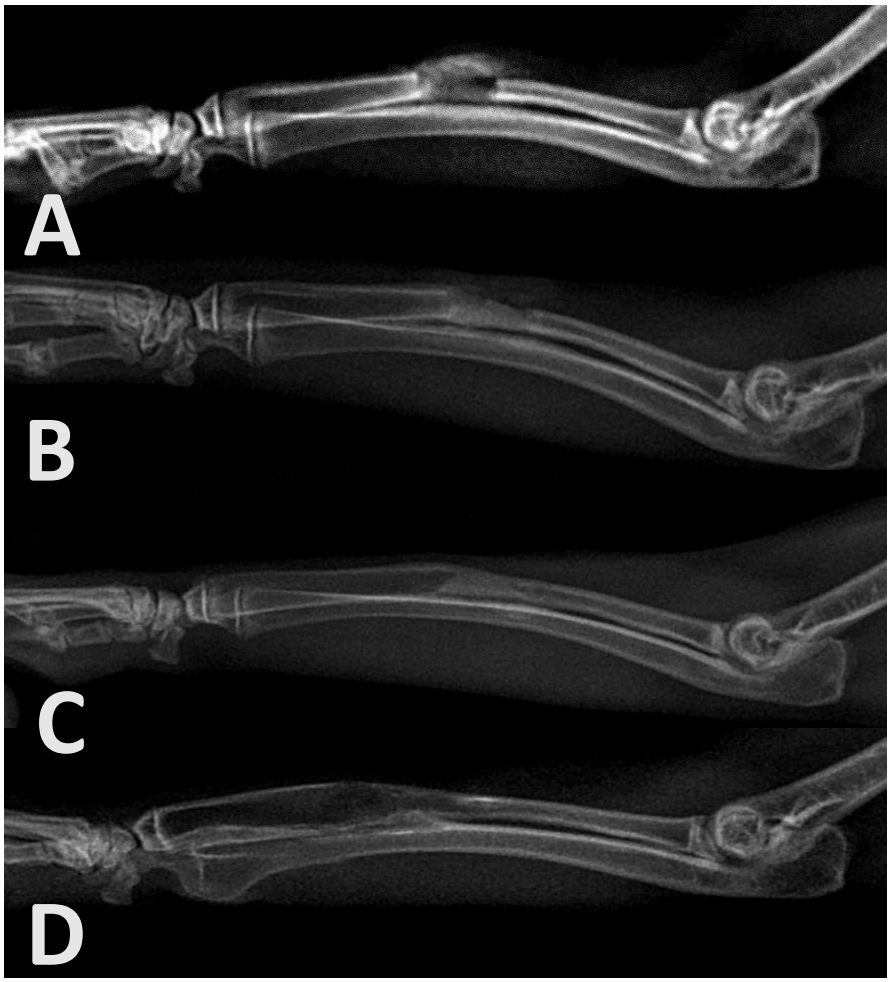

Fig. (5): showing radiographic images of control group at 2nd week (A), 4th week (B), 8th week (C) and 12th week (D).

DISCUSSION

In this study, CCNPs derived from Coral (stony coral) was used as biomaterial because its structure resembles the mineral structure of the normal bone, also it was easily prepared, cost effective and had no toxic effect on the animal model (Mahmood et al., 2017). Coral itself used as used as bone scaffolds in many researches to ascertain an upgraded bone ingrowth and osseointegration making it an effective osteoconductive biomaterial (Parizi et al., 2013). Although, most of studies revealed that coral has no osteo-inductive properties (Misk et al,2020).

In our study, AgNPs were used as it have multi-potential effect, it acts as antibacterial, antifungal, antiviral, anti-inflammatory and have good osteogenic effect (Sadeghi et al., 2012). Other reasons for using AgNPs its availability, easily prepared, cost effective (Brennan et al., 2015).

In this study, A-PRF was used, as a fibrin biomaterial as it brings the favorable components present in a blood sample such as a large quantity of platelets, cytokines and leukocyte (Toffler et al., 2009). Also, PRF offers the following four advantages: First, PRF plays a valuable mechanical role in conserving and serving the grafted materials. Second, the platelet cytokines (IGF-1, TGF-β, and PDGF) are gradually released as the fibrin matrix is resorbed. Third, the fibrin network at the regenerative site eases vascularization, cellular migration and continued existence of the graft, thus forming a continual process of healing. Finally, the presence of cytokines and leukocytes in the network of fibrin can play a significant role in the self-regulation of inflammatory and infectious phenomena within the grafted material (Simonpieri et al., 2009; Kökdere et al., 2015).

A-PRF and PRF shares many similarities (shape, consistency, biochemical properties), but it was found that A-PRF releases a higher GFs than PRF, this is because the protocol of preparing both A-PRF and PRF, in which A-PRF is prepared at low speed of centrifugation (1500 rpm) for longer time (14 minutes) comparing to PRF (3000 rpm for 10 minutes), making the A-PRF preserves higher levels of platelets and cytokines (Titirinli et al., 2017).

Radiographically, the radiographic imaging showed that adding bone graft substitutes to critical segmental radial defect with length 14 mm have great radiographic changes. The statistical analysis showed significant differences in bone formation, proximal cortical union, remodeling and total radiographic score (at 2,4,8 and 12 weeks of observation period) among the two experimental groups ($P < 0.05$). In control group, at the end of the observation period (12 week) bone regeneration was limited to the vicinity of the cut edges of the defects. No bony union was observed radiographically in any animal. Same data was reported by (Meng et al., 2019) as they found limited new bone growth without any bony union.

On other hand, CCNPs/AgNPs/A-PRF groups showed complete healing at end of period of observation with complete remodeling (canalization). This data agreed with **Salih, et al., (2018)** who revealed that using a combination of PRFM and AgNPs together gave better acceleration in the bone healing process than using each one of them separately.

CONCLUSION

All of the above makes CCNPs/AgNPs/A-PRF composite a perfect mixture for bone healing acceleration as it contains osteoconductive (CCNPs), esteogenic (AgNPs) and esteoinductive (A-PRF). This study is considered as Prelude to use CCNPs/AgNPs/A-PRF comp

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