Studies on A-PRF and A-PRF/Coral powder for Reconstruction of Induced Bone Defects in Dogs

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ABSTRACT

Activated platelets-rich plasma (A-PRP) was used extensively in various experimental and clinical models. However, the use of anticoagulant in A-PRP preparation may interfere with healing process. Second generation of A-PRP were developed without adding anticoagulants or activators which where know as platelet rich fibrin (PRF). The PRF was improved during preparation and were known as advanced-PRF (A-PRF). *In vitro* studies, A-PRF showed releasing of significantly higher growth factors over time than PRF however, limited studies document its efficiency *in vivo.* Stony coral was used as a bone substitute for its osteo-conductivity. It can be used either alone or a part of a composite with reported success and low complication rates. The aim of the present study to evaluate the efficacy of A-PRF alone or with combination. Twelve adult mongrel dogs (15-25 kg body weight) of both sexes were used in this experiment. A 3 drill hole defects of 10 mm diameter were made in the tibial tuberosity 1 cm apart. The upper hole was filled with A-PRF alone, the middle hole was left empty as control and the lower hole was filled with mixture of A-PRF & coral powder. Sequential radiographs were obtained every week for the first month and every two weeks until completion of the study at 12 weeks. Radiographic opacity score of the defect compared to adjacent bone range from -3 to +3 and radiopacity score of host-implant interface which ranged from 0 to +3. Dogs were euthanized at the end of respective observation period at 2nd, 4th, 8th and 12th week post-operative for histological evaluation using *Emery scoring system.* By the end of the study, using A-PRF and with or without combination of coral powder was significantly improved and accelerate bone healing. Addition of coral powder scientifically improves the quality of remodeled bone histologically however, there was no significant radiographic differences.

Keywords: *A-PRF, PRP, Coral, Bone healing.*

INTRODUCTION

Bone is a highly specialized tissue with high regeneration capacity. However, defects due to trauma, infections, cysts and tumors may decrease the regeneration ratio and can lead to healing problems (Hollinger, *et al.,* 1996). Thus, searching for bone graft substitutes become a critical need. Graft materials were used to facilitate and accelerate bone formation and increases the physical endurance of bone defects (McAllister, *et al.,* 2007). Bone substitutes work by one of three mechanisms: osteogenesis, osteoinduction or osteoconduction. The ideal bone substitute that offers these three properties is the autologous bone graft ''gold standard'' (Lynch, *et al.,* 2008). However, the clinical application of autologous bone grafts is sometimes limited due to requiring an additional surgical harvesting site (Barone, *et al.,* 2007). Alternative bone substitute such as allograft, xenograft or other biomaterials were used to overcome the drawback of autologous

grafts (Barone, *et al.,* 2007 & Johansson, *et al.,* 2010).

In the past decades, studies revealed that platelets are responsible of releasing important biomolecules that are capable of stimulating cell activation and proliferation involved in healing process (Khan, *et al.,* 2000). 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 (Del Corso, *et al.,* 2012). In orthopedics, activated platelets-rich plasma (A-PRP) was used extensively in various experimental and clinical models. However, the use of anticoagulant in A-PRP preparation may interfere with healing process, so a second generation of A-PRP were developed without adding anticoagulants or activators (thrombin or calcium chloride) which where know as platelet rich fibrin (PRF) (Dohan, *et al.,* 2006). The PRF were improved by decreasing the speed and prolonging the centrifugation time which were known as advanced-PRF (A-PRF) (Dohan, *et al.,* 2006 & Ghanaati, *et al.,* 2014). *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*. Stony coral was used as a bone substitute for its osteo-conductivity. It can be used either alone or a part of a composite with reported success and low complication rates (Ghanaati, *et al.,* 2014).

The present study aims to evaluate the in vivo efficacy of A-PRF and with/ or without its combination with coral powder as a bone graft substitute for acceleration of bone healing in a dog model.

MATERIALS AND METHODS

Animal model and surgical procedure

Twelve adult mongrel dogs (15-25 kg body weight) of both sexes were used in this experiment. The protocol was approved by the Animal Care and Use Committee, Sadat City University College of Veterinary Medicine, Sadat City, Egypt. Dogs were premedicated with atropine sulfate (Atropine sulfate® : 0.1 % sol. Med. Co., A.R.E.) at dose of 0.04 mg/kg bw S/C and xylazine (Xylaject® : 2% sol. ADWIA Co., A.R.E.) at dose of 1 mg/kg bw

I/V, general anesthesia was induced by ketamine hydrochloride at dose rate 10 mg/kg bw I/V and maintained by using 2.5 % sol. thiopental sodium (Anapental® : SIGMA Co., A.R.E.).

A 10 cm skin incision was made at craniomedial aspect of hind limb. The incision started just caudal to tibial tuberosity extending downward. Three holes with 10 mm in diameter with 1 cm apart were made by driller with driller bit 10 mm under continuous saline irrigation. The upper hole was filled with A-PRF alone, the middle hole was left empty as control and the lower hole filled with mixture of A-PRF & coral powder. Periosteum and subcutaneous tissue were sutured by simple continuous pattern using 3-0 polyglactin 910 (vicryle®), finally skin was sutured by vertical mattress pattern using silk. Stiches were removed after 12 days.

A-PRF preparation

A-PRF was prepared as described by Kobayashi, *et al.,* (2016) a 20 ml of whole blood obtained from jugular vein by using 20 ml sterile syringe with needle 21G. The collected blood distributed in four sterile glass tubes (5 ml for each) without adding anticoagulant. The blood sample were centrifuged in a laboratory centrifuge (Unico; united products & instruments INC; USA) at 1500 rpm for 14 min at room temperature. A fibrin clot was formed at the middle of the tube between the red corpuscles at the bottom and a cellular plasma at the top.

Coral powder preparation

Coral fingers (*porites sp.*) were cut into small pieces, each part was rasped to remove any suspended impurities, then coral fingers were crushed into small particles with diameter 0.5-1 mm. Coral powder was packed in double wrapped plastic roll and sterilize by autoclaving (121 c° for 20 minutes) (Irigaray, *et al.,* 1993).

Radiographic evaluation

The sequence of bone healing in terms of filling of hole defects with bone deposition was monitored via sequential radiographs taken immediately post operation and every week for the first month and every two weeks until completion of the study (12 weeks). An x-ray apparatus (Siemens 300) was used to take a medio-lateral view on 18x24 cm film at 52 KV, 15 mAs and 80 cm focal film distance.

The radiographic images were evaluated by two radiologists (without previous information). Two scores were designed, the first score according to radiographic opacity of the hole defect compared to radiographic opacity of the bone, the score range from -3 to +3. The second score was evaluated according to radiopacity of host-implant interface which ranged from 0 to +3 (sharshar, 2012).

Histological evaluation

Euthanasia was performed at the end of respective observation period of each defect at (2nd, 4th, 8th, 12th week). The operated tibia was harvested and examined grossly for signs of bone healing. Bone specimens placed on 10% buffered formalin for 72 hours then decalcified by 10% EDTA di sodium (ethylenediamine tetra-acetic acid di sodium salt) for one month, EDTA changed weekly until complete decalcification. Sections of 5 μm thickness were obtained and stained with Hematoxylin & Eosin stain and covered with cover slips (González, *et al.,* 2013). Sections were examined under light microscope with objective lenses 200×, 400× magnifications and blindly scored by two pathologists, the defects were evaluated according to *the Emery scoring system* (Emery, *et al.,* 1994); the gap was empty (score 0)**,** if the gap was filled with fibrous connective tissue only (score + 1), with more fibrous tissue than cartilage (score + 2), more cartilage than fibrous tissue (score + 3), cartilage only (score + 4), more cartilage than bone (score + 5), more bone than cartilage (score + 6) and filled only with bone (score + 7).

Statistical analysis

All the results were reported as mean, SEM using two-way ANOVA statistical analysis. $p \leq 0.05$ was considered as statistically significant. All the values were analyzed using Prism 5 for windows software *(version 5.01, 2007).*

RESULTS

All dogs tolerated the surgical procedure during the course of the study. No infection or inflammation was observed at the surgical site.

Radiographic findings

During the first four weeks of the study there was a high significant difference between A-PRF& coral mixture group and other groups. At the end of the third week, the corals powder stars to resorb while radio-opacity increases in other groups. By the end of the 4th week, A-PRF group showed non-significant increase in radio-opacity than A-PRF/coral mixture group. After the 4th week post-operative, the A-PRF& coral mixture showed a non-significant improvement over A-PRF and control group till the end of observation period (Figure 1 & Table 1).

The hole defects in both A-PRF and control group appeared radiolucent at two weeks post-operative. Starting from the fourth week, the defect appeared radiolucent at its center and radiopaque at the periphery. From the 8th weeks till the end of observation period, there was a gradual decrease of the radiolucent area at the center of the defects in A-PRF defect while in control defect the size of centered radiolucent area remains almost constant.

The hole defects in A-PRF & Coral mixture group were completely radiopaque at two weeks post-operative. At the third week, marked radiolucency started to appear at the center of the defects. From the 4th week till the end of observation period there was a gradual decrease of the radiolucent area at the center of the defects till become almost disappeared by 12 weeks post-surgery.

Histopathological findings

The A-PRF and A-PRF& coral mixture defects showed non-significant improvement over control defect by 8 weeks post-surgical intervention. However, by the twelve week the A-PRF& coral mixture showed significant improvement over control defect and a non-significant improvement over A-PRF defect (Figure 2 & Table 2).

Control group showed immature unorganized fibrous connective tissue with blood vessels at two weeks post-operative. At fourth and eight-weeks, the fibrous connective tissue became organized and denser. By the twelve weeks, the defects were filled with dense fibrous connective tissue and fibrocartilage. (figure 3)

A-PRF group showed immature unorganized fibrous connective tissue with blood vessels at two weeks post-operative. At fourth week, the fibrous connective tissue became denser and more arranged in hyaline like matrix. At eight weeks, a newly formed cartilage started to appear and by the twelve weeks the defect showed newly formed bone with osteonal canal lined with osteoblasts. (figure 4)

A-PRF & Coral mixture group showed immature unorganized fibrous connective tissue with blood vessels at two weeks post-operative. At four weeks, the fibrous

connective tissue became denser with presence of osteoblasts arranged in epithelioid manner lining the compact (host) bone. At eight weeks, chondrocytes appeared and located in lacunae and surrounded by collagenous matrix, also the defect showed newly formed bone with osteonal canal filled with osteoblasts. At twelve weeks, newly formed bone became mature and more compacted with multiple haversian systems (figure 5).
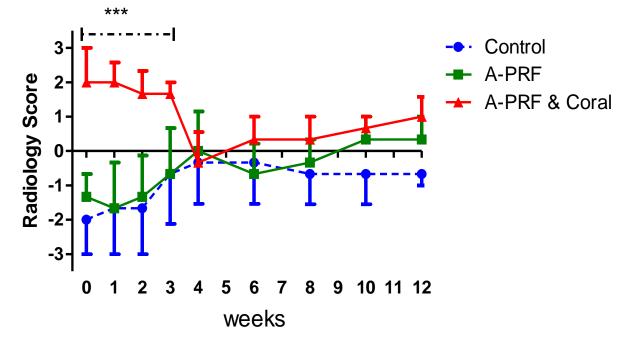

Fig. (1): Radiographic score for the three defects for 12 weeks follow-ups. *** high significant deference's *at P < 0.05*.

Table (1): mean radiopacity of control, A-PRF, A-PRF/Coral defect areas in sequential radiography:

Period of observation	Control defects	A-PRF defects	A-PRF+ coral defects
Zero day	-2 a	-1.333 a	+2 b
1 W	-1.666 a	-1.666 a	+2 b
2 W	-1.666 a	-1.333 a	+1.666 b
3 W	-0.666 a	-0.666 a	+1.666 b
4 W	-0.333	0	-0.333
6 W	-0.333	-0.666	+0.333
8 W	-0.666	-0.333	+0.333
10 W	-0.666	+0.333	+0.666
12 W	-0.666	+0.333	+1

At the end of the study there was no significant difference *(P > 0.05)* between control defect and A-PRF defects and A-PRF/ Coral defects.

Table (2): Showing the histological score evaluation of control, A-PRF, A-PRF/Coral defect areas:

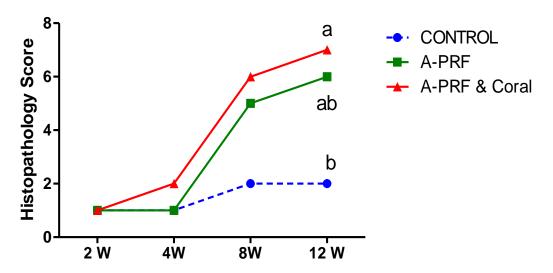| Defect obs. period	Control defect	A-PRF defect	A-PRF & coral defect
2 weeks	+1	+1	+1
4 weeks	+1	+1	+2
8 weeks	+2	+5	+6
12 weeks	+2a	+6 ab	+7 b

a significant difference at *P < 0.05*.

Fig. (2): histological score for the 3 defects for three months, a significant difference at P< 0.05 between control defect and two other defects
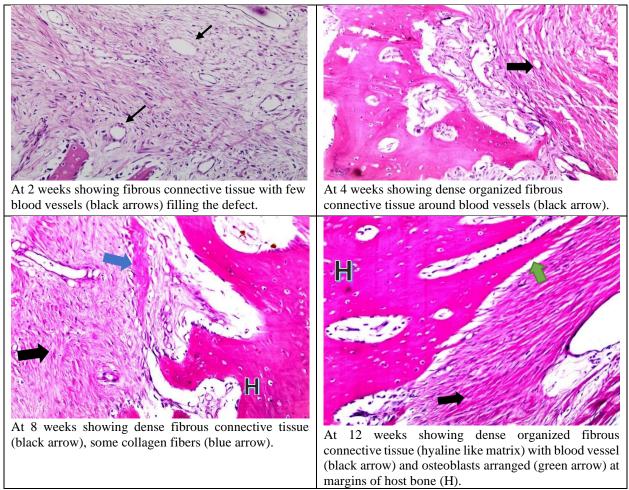

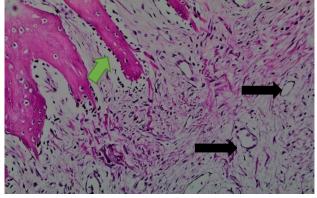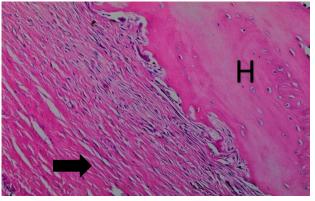|	
At 2 weeks showing fibrous connective tissue with few blood vessels (black arrows) filling the defect.	At 4 weeks showing dense organized fibrous connective tissue around blood vessels (black arrow).
At 8 weeks showing dense fibrous connective tissue (black arrow), some collagen fibers (blue arrow).	At 12 weeks showing dense organized fibrous connective tissue (hyaline like matrix) with blood vessel (black arrow) and osteoblasts arranged (green arrow) at margins of host bone (H).

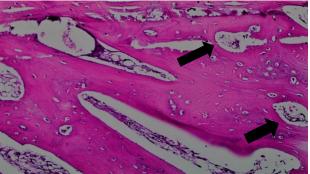**Fig. (3): Histological picture of control defect areas over time**

At 2 weeks showing fibrous connective tissue around few blood vessels (black arrows) and osteoblast (green arrow) lining the compact bone.

At 4 weeks showing dense organized fibrous connective tissue arrange in hyaline like matrix (black arrow)

At 8 weeks showing hyaline like cartilage (black arrow) and number of osteoblast lined osteonal canal of newly formed bone (yellow arrow).

At 12 weeks showing osteonal canal filled with osteoblasts (Black arrows).

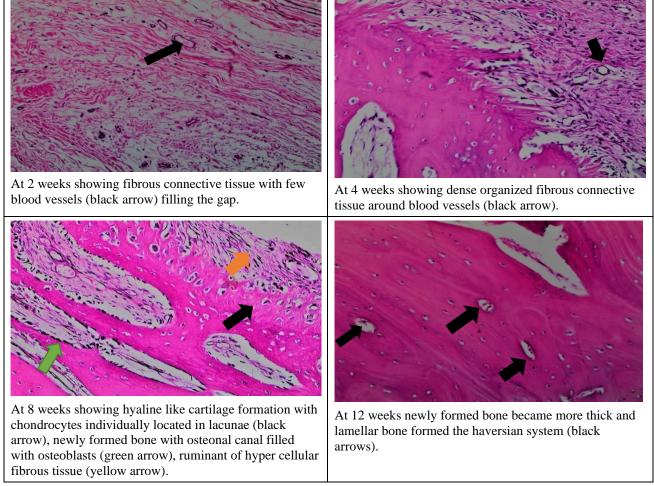**Figure 4. histological picture of A-PRF defect areas over time**

At 2 weeks showing fibrous connective tissue with few blood vessels (black arrow) filling the gap.

At 4 weeks showing dense organized fibrous connective tissue around blood vessels (black arrow).

At 8 weeks showing hyaline like cartilage formation with chondrocytes individually located in lacunae (black arrow), newly formed bone with osteonal canal filled with osteoblasts (green arrow), ruminant of hyper cellular fibrous tissue (yellow arrow).

At 12 weeks newly formed bone became more thick and lamellar bone formed the haversian system (black arrows).

Figure 5. histological picture of A-PRF/Coral powder mixture defect areas over time.

DISCUSSION

The objective of this study was to evaluate the efficacy of A-PRF and coral as a two bone graft substitutes to induce new bone formation and accelerate bone healing. Using dogs as an animal model in this study because of similarities of bone structure and biological repair process to those of human (Shafiei, *et al.,* 2012).

Radiographically, during the first 3 weeks, the radio-opacity of the A-PRF & coral mixture was highly significant (P<0.0001) from other defects. These results do not indicate the superiority of this group over other groups however, the presence of calcium in coral powder is the reason giving false impression that the defect is sealed.

From the 4th week to the end of the study, A-PRF and A-PRF/coral mixture groups showed better radio-opacity than control group however, these opacities were no significant (P > 0.05) between the three groups. Same data was reported by Pripatnanont, *et al.,* (2013) as they found no significant difference between PRF defects and control defects radiographically, likewise (Faot, *et al.,* 2017) who found no significant difference between PRF defects and control defects using micro-CT. One of the study limitations that our radiographic findings may be less precise as we used traditional radiography however nowadays digital radiography has become a much more efficient.

In contrast, Shafiei, *et al.,* (2012) reported that radiological results showed that bone healing was enhanced when human PRP (hPRP) was used alone in comparison with hydroxyapatite-hPRP or coral-hPRP when used in acritical defect in rabbits, although (Mooren, *et al.,* 2007) reported that PRP was unable to induce bone healing in early and late stage in goats.

Histologically, by the end of the study there was no significant difference between A-PRF and A-PRF/Coral Mixture. However, a significant difference was found between A-PRF/coral mixture and control group. A-PRF/coral powder mixture group has good efficacy to induce new bone formation and accelerate bone healing than in control defect.

There was nearly no significant difference in control defects in healing progression along the period of observation, on the other hand, there was more bone healing progression at eight and twelve weeks than two and four weeks in A-PRF alone and A-PRF/coral powder. These results were agreed with (Pripatnanont, *et al.,* 2013*)* as they mentioned that PRF had a positive effect on bone formation after eight weeks when used alone or combined with autogenous bone. In another study, Bölükbaşı, *et al.,* (2013) found a histomorphometry increase in bone formation with the addition of PRF to biphasic calcium phosphate in a surgically induced defects in sheep tibia. Another study , in the same side Pradeep, *et al.,* (2012) mentioned that hydroxyapatite increase the efficacy of PRF when used to treat intra-bony defect in humans [19]. On the other hand, Kökdere, *et al.,* (2015) found no significant difference in histological picture of PRF at 4- and 8-weeks post-operation when was used to fill an induced hole defect in tibial bone in rabbit model.

CONCLUSION

From the results of this experimental study, we can conclude that A-PRF has good efficacy in bone generation, addition of coral powder had a radiographic non-significant improvement over A-PRF defect however, the mixture had a significant improvement histologically from control group by the end of the study.

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