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Evaluation of use of platelet rich plasma (PRP) with decellularized caprine aortal xenograft for healing of critical size bone defects in New Zealand white rabbits

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Abstract
The present study was conducted to evaluate the effect of platelet rich plasma (PRP) in healing of critical sized bone defect in New Zealand white rabbits. Caprine aorta was decellularized by an herbal decellularization agent soapnut pericarp (*Sapindus mukorossi)*. Further prepared aortal scaffold was implanted in critical size defect in radius bone. Rabbits were divided in two groups. Group I was implanted with scaffold alone, Group II was implanted with aortal scaffold and platelets rich plasma. The analysis was done by histology, serum calcium, phosphorus and alkaline phosphatase levels, ontogenesis by radiography. Radiographic score shows better healing in PRP treated group. Biochemical studies shows alkaline phosphatase level came to near normal level in group II at 12 weeks. Overall, from analyzing parameters it was found that Group II (treated with aortal scaffold and PRP) was most efficient in bone healing than group I (aortal scaffold alone).This concludes that adopted method requires certain modification for complete bone healing in given time.

Keywords: Bone defect, Decellularization, PRP, radiography, aortal scaffold

Introduction
Bone grafting has become popular surgical procedure for treating bone deficiency (Gardin *et al.*, 2012) [4]. Bone defects due to trauma, pathological and physiological bone resorption represent a major challenge and are a global health problem, in both human and domestic animal individuals (Campos *et al*, 2018) [5]. Decellularization refers to the removal of all cellular and nuclear material with minimal impact on the extracellular matrix's composition, biological activity, and mechanical integrity (Gilbert *et al.*, 2006) [5]. The use of bone grafts in treating non- or delayed unions as the result of large bone loss is well established. However, despite good outcomes, the time to achieve complete union is still considerably long. To overcome this problem, the use of platelet-rich plasma (PRP) has been advocated albeit with varying success (Kanthan *et al*, 2011) [7]. PRP may provide optimistic prospects for bone graft procedures. Platelets contain growth factors. Growth factors are protein signaling agents, which are pleiotropic, causing multiple biological effects; they are expressed during different phases of bone repair. Many of these factors are known to play a role in the differentiation of mesenchymal progenitor cells to specific lineages such as chondroblast and osteoblast; thus, they can be useful in improving the healing processes. Therefore, the potentiality of allograft transplantation with PSC and growth factors needs to be investigated further for general application.

Materials and Method
Sample preparation
The caprine aorta was cleaned under aseptic condition using sterile phosphate buffered saline (PBS) with antibiotic amikacin (0.1 mg/mL) and protease inhibitors immediately after collection (0.2025 percent EDTA. Decellularization of native caprine aorta tissue was done by 100 mL of soap nut pericarp extract for 120 hours on an orbital shaker.

Isolation of platelet rich plasma from blood
A total of 5 ml of blood was drawn from the central vein of the ear in rabbits placed into Acid citrate dextrose (ACD) vacationers. Centrifugation of the blood to separate the plasma from the cellular products was performed for 20 min at 150 G.

The supernatant were aspirated and centrifuged for a further 10 min at 450 G. The forming bilayer of plasma containing the upper platelet-poor plasma and a lower layer of PRP was then separated using an aspirator. Only the lower layer was used for this experiment. Each vial of prepared PRP was sampled and sent to diagnostic laboratory of our college for platelet count to ensure that high concentrations of platelets were obtained in the prepared plasma. Samples obtained from the individual rabbits, it was found that the platelet numbers were between 2.62 and 4.6 times higher than that of the peripheral blood. PRP obtained from the rabbits were strictly administered into the corresponding rabbit from which the peripheral blood was obtained, that is, as an autologous biological product. Prior to injecting into the defect site, calcium chloride and bovine thrombin were mixed with PRP in a ratio of 1:6 for platelet activation (Kanthan *et al.*, 2011) [7].

Animals and surgical procedure

Experimental procedure was done after prior approval from Institutional Animal Ethics Committee (IAEC/CVSc/2/P-05/2020/15). Bone grafting performed on 12 New Zealand White rabbits, weighing about 2.5±0.5 kg and of 18±6 months procured from the Scientific Animal House, Ayodhya, Uttar Pradesh. Two groups were made and animals allotted to these groups according to type of implantation. There were six New Zealand White rabbits in each group. Animals were acclimatized for a period of 15 days before start of the study. They were housed under a natural light cycle: 12 h light 12 h darkness, at 22 C at the Animal House for Experimentation, Department of Veterinary Surgery and Radiology, throughout the experimental period (Abid *et al.*, 2019) [1]. All surgery was performed under ketamine hydrochloride and Xylazine hydrochloride anesthesia.

Table 1: The osteotomy gap of animals of different groups was treated as follows

Group	No. of rabbits	Treatment
I	06	Decellularized aorta
II	06	Decellularized aorta + platelet rich plasma (PRP)

Pre-operative preparation and Anesthesia

The surgical site of forelimb was shaved and prepared aseptically with 10% providence-iodine. Ketamine hydrochloride at the rate of 60 mg/kg and Xylazine hydrochloride at the rate of 6 mg/kg by intramuscular injection were used to anesthetize the animals.

Surgical procedure

A 5 cm longitudinal incision of skin and subcutaneous tissue in the middle third of the radius (cranio-medially) was made and exposes the muscle. Dissect the space between extensor and flexor muscles groups so that there was wide view of radial bone. The periosteum was incised and reflected and a Hohmann retractor was placed between the radius and ulna to protect the ulna after exposing bone. In the mid shaft of the radius a gap was created by removing 1.5 cm of bone which was cut with the help of heck saw. The periosteum around the osteotomy site was removed. Good amount of the decellularized aortic tissue were applied to fill the created bone defect. Muscle and subcutaneous tissue were sutured with continuous suture pattern using vicryl suture and then skin was sutured with interrupted suture pattern using silk suture. Bone defect was grafted with decellularized aortic tissue and 0.5ml of a PRP 8 lakhs platelets/ ml which was initially collected, was injected directly into the fracture focus using a micropipette in group II.

Evaluation of fracture healing efficiency of decellularized caprine aorta scaffold with Plate rich plasma (PRP) by following parameter

1. Gross observation

Reconstructed radiuses were harvested after sacrifice and completely cleared from the soft tissues. The status of callus growth, degradation and bone healing and new bone formation at the bone graft in the radius was observed.

2. Histological analyses

Sample from the bone graft sites of the radius was collected at 6th and 12th week post-surgery and fixed with 10% paraformaldehyde. The samples were demineralized in 20% formic acid for 7 days, dehydrated in alcohol with increasing concentrations (70% -100%) for 1hour each, cleared in two changes of xylene for one hour each and were paraffin embedded at 60°C for 1 h. 5 micrometers sagittal sections were cut and stained with hematoxylin and eosin and Masson's trichrome. The micrographic images from the light microscope were quantified.

3. Radiological assessment

Radiographs were taken at 55 kVp, 6.5 mAs, 85 cm FFD and mediolateral position to evaluate fracture healing and bone callus formation. The operated site was radiographed just after operation and subsequently on 6th week and 12th week after the surgery. Assessment of new bone (NB) formation and remodeling was based on the modified Lane and Sandhu (1987) [9] radiological scoring system.

4. Biochemical Parameters

Blood samples (2 ml) was collected in the morning 1 to 2 h before surgery (time 0) and at 3, 6 and 12 weeks after surgery for evaluation of serum calcium, phosphorus and alkaline phosphatase.

Statistical analysis

The data was presented as mean and standard deviation. The Student's t-test was performed to compare the difference between the mean values of different groups. Differences at a level of $p < 0.05$ were considered to be statistically significant.

Result and Discussion

In present study platelets rich plasma (PRP) treated group showed better healing result compare to other non PRP treated group. Thus the present study was undertaken to objectively quantify the effect of PRP on autogenously bone grafts of radius bone in a rabbit model. Platelet counts confirmed that the PRP preparation technique used in this study produced a source of highly concentrated platelets. In an animal pilot study, Aghaloo *et al.* (2004) [2] used PRP in rabbit cranial bone defects with groups consisted of bone graft, bone graft with PRP, PRP alone, and no treatment group. Defects were evaluated Radiographically and

histologically at 1, 2, and 4 months postoperatively. Results did not show a significant difference for defects treated with the addition of PRP. When PRP is used in conjunction with a bone graft, very less healing occurs, making the addition of PRP a remaining questionable clinical decision Miloro *et al.* (2010) [10] unlike present study.

Anesthesia, defect creation and treatment

Twelve (12) New Zealand white rabbits were randomly divided into two groups of six, each consisting of six animals of roughly equal age and weight. The forelimb slightly below the elbow joint was prepared. Xylazine (6 mg/kg body weight) and ketamine (60 mg/kg body weight) intramuscular injections were used to anaesthetize all of the animals (Udehiya *et al.*, 2013) [11]. Immediately after collection and isolation of platelets rich plasma (PRP) surgical procedure for creation of bone gap started so as to apply freshly collected cells at the osteotomy site (Kanthan *et al.*, 2015) [7]. Muscles and fascia were separated after skin incision, then radius bone was exposed and in diaphysis of radius bone an osteotomy gap of about 1.5 cm was created by hacksaw. The animals recovered within 1 h after surgery and were able to sit in normal posture within 24 h. All wounds were healed within 1 week without any observable inflammation and infection. There was no evidence of infection or other complications in any animal. These results were in consistent with Zhao *et al.* (2020) [12].

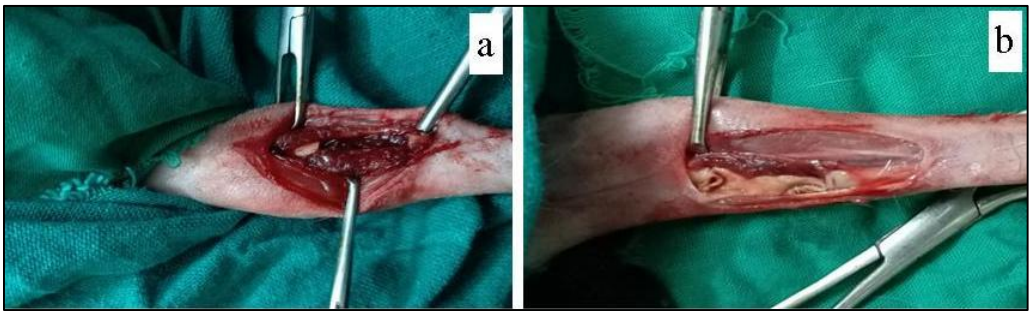

Fig 1a: Creation of defect in radius bone of New Zealand white rabbit and **b.** transplantation of caprine decellularized aortic graft

1. Gross examination

Gross observation bone defect of revealed that in group II there was complete absorption of aortal scaffold and bridging of two end of bone. In group I, aortal scaffold was not completely absorbed and non-union of bone with no callus at 12 weeks. Zhao *et al.* (2021) [13] discovered that all sizes of bone defects in Groups A (tissue-engineered periosteum) and Group C (tissue-engineered periosteum with allogeneic deproteinized bone) group B all showed full union (allogeneic deproteinized bone). Group D faults (small intestine Submucosa-defective stem cells) did not heal.

2. Histomorphology

H&E staining was used to perform a histopathological study on samples of decalcified bone. In group I, we saw the least degree of bone repair. These tissues lacked callus and granulation tissue. The vasculature and bone marrow were injured in group I. Histology showed that group II had early new woven bone development. Around the periphery of defect, new layers of woven bone developed. In group II, there was an early callus that contains many cells which resemble chondrocytes and fibroblasts. The presence of inflammatory cells suggests that bone healing was taking place. The gap areas seen in fig. 2 contained very little fibro cartilaginous tissue and little graft-bone interface. The presence of osteoplastic activity suggests bone repair similar to that shown in Kanthan *et al.* (2011) [7].

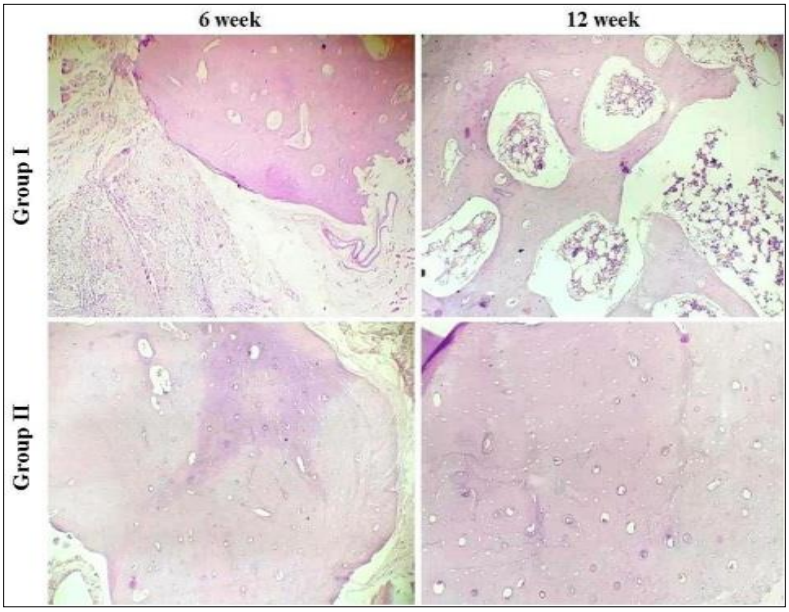

Fig. 2: Histological evaluation of HE staining in Group I (decellularized aorta scaffolds) and group II (decellularized caprine aorta with PRP at 6 and 12 weeks interval after implantation)

3. Radiographic examination

Radiographic images of the animals in groups I (Decellularized aorta scaffolds) and II (Decellularized caprine aorta with PRP) at intervals of 0, 6, and 12 weeks are shown in Fig 3. At six weeks after surgery, low density calluses were seen at the borders of bone with the defect region in Group I. In comparison to 6 week interval, more calluses were seen after 12 weeks, and newly produced bone and bone mineral density increased. Six weeks after surgery, in Group II, the elevated callus quantity was evident. At 12 weeks, the location of the bone deficiency had much more newly produced bone. Under X-ray inspection, fresh bone growth was seen while the scaffold was largely destroyed. The results of the present study illustrated that formation of new bone tissue in Group II was better as compared group I.

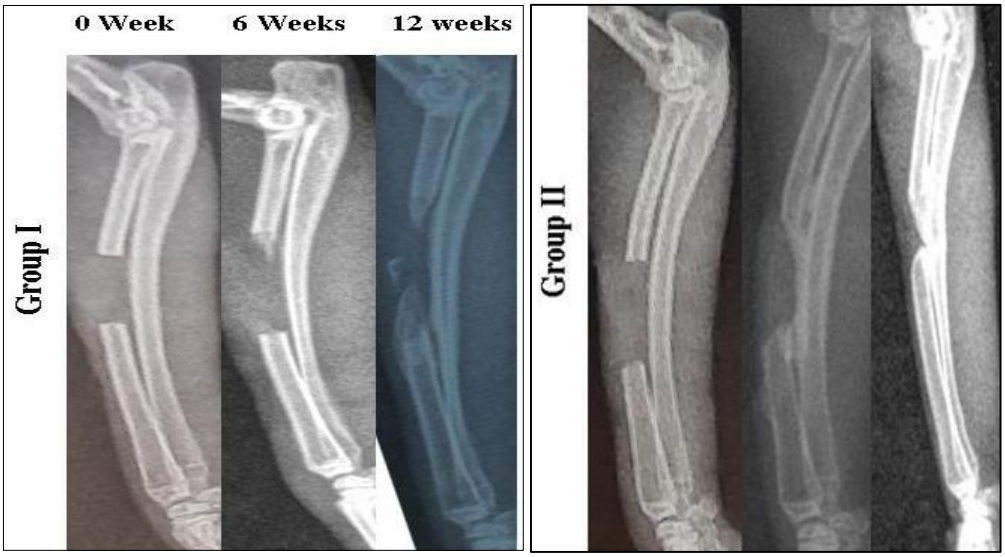

Fig 3: Radiographic images of group I (decellularized aorta scaffolds alone) and group II (decellularized caprine aorta with PRP) at 0, 6 and 12 weeks interval after implantation.

4. Biochemical Studies

The serum alkaline phosphatase level was significantly lower on week 12th post operatively in group I, except in group II at 3rd week. There was gradual decrease of serum alkaline phosphatase value up to post-operative day 60 was consistent with findings of Lal and Jacob (1976) [8].

Table 2: Mean ± SE of alkaline phosphatase (KA units) of animals of different groups at different time intervals

Parameter	Group	Time interval (Weeks)			
		0	3	6	12
Serum ALKP	I	4.45±0.03[a]	3.61±0.11*[a]	2.44±0.16*[a]	1.64±0.13*[a]
	II	6.96±0.25[b]	7.43±0.14[b]	5.41±0.15[b]	4.40±0.08*[b]

*Differ significantly from day 0 value

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