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RESEARCH ARTICLE

Histopathological evaluation of the osteogenic activity of autologous platelet-rich plasma in canine caudolateral ulna defect model

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Abstract

Variety of products and surgical approaches to stimulate biologic factors and promote osteogenesis have been developed over the years, but surgeons still face challenges relating to bone healing. This study was designed to evaluate the histopathologic effects of autologous platelet-rich plasma and cancellous bone grafts on caudolateral ulna ostectomy of Nigerian local dogs. Twelve healthy Nigerian indigenous dogs (males) were used for the study. They were randomly grouped into four viz: Platelet-rich plasma-treated group (PRPG) (n=3), cancellous bone graft-treated group (CBG),(n=3), platelet-rich plasma/cancellous bone graft-treated group (PRP/CBG),(n=3) and Normal saline treated control group (CG), (n=3). Caudolateral ulna defects were created on the left ulna of each dog under general anesthesia and treated as follows: The PRPG were treated by application of autologous PRP on the defects prior to closure; the PRP/CBG defects were treated by applying both PRP and cancellous bone grafts on the defects; CBG were treated with autologous cancellous bone grafts alone while the control defects were treated by applying normal saline on the defects prior to closure of the soft tissues. Animals were euthanized at week 10 and the bridging callus of the defects analyzed by histopathology. The PRP treated defects either in combination with cancellous bone grafts or alone demonstrated more matured lamella bone compared to the CBG and control defects. The control defects were characterized with fibrocartillagenous zones of intense cellular activities mainly of chondroblasts and oeteoblasts. The mean histologic indices of the PRPG and PRP/CBG defects varied significantly ($p < 0.05$) with the CBG and the control defects. The results showed that the PRP has a positive osteogenic effect in treatment of caudolateral ulna ostectomies of dogs but its activity was optimal when combined with autologous cancellous bone grafts.

INTRODUCTION

Researches into the biology of bone, ligament and tendon healing have led to the development of variety of products designed to help stimulate biologic factors and promote healing (Foster *et al.*, 2009). The use of exogenous recombinant proteins including bone morphogenic proteins is being investigated. Application of mechanical vibration along the axis of the fractures, ionic resonance electromagnetic field stimulation, and static magnetic force with Samarian cobalt magnets has all been employed. In our rural setting, many herbs have been investigated with varying degree of successes and failures. Previous studies on the repair of segmental defects have focused on bone

matrix substitutes (Grundel *et al.*, 1991Delloye *et al.*, 1992; Gogolewski *et al.*, 2000). However, these substitute matrices do not perform as well as autograft for several reasons including histochemical responses by the host tissue and a dearth of living cells. Enhancing the osteogenic capabilities of these bone matrix substitutes for the treatment of segmental defects is currently an active area of research (Hobbenaghi *et al.*, 2014).

In spite of these, surgeons still continue to face challenges relating to the healing of bone. There still abound many cases of delayed and non union fractures in many orthopedic clinics all over the world. This results in increased morbidity and financial burden on the patients or the clients.

Platelet- rich plasma has been used to treat soft tissue wounds since 1985 (Driver *et al*., 2006).

It is a growth factor agonist (Petrova and Edmonds, 2006) with both mitogenic and chemotactic properties (Millington and Norris, 2000; Marx, 2001).It has been incriminated to enhance osteogenesis. .Its potential for enhancing osteogenesis in bone defect models is however controversial as a result of variability in the results obtained, probably because most of them were case reports which lacked controls and were of small sample sizes.

In this work, the osteogenic potential of autologous cancellous bone graft and platelet rich plasma was histologically evaluated in a randomized control study conducted in Nigerian indigenous dogs.

Methods

Ethics statement

This work was carried out in accordance with the guidelines for animal experiments released by the National Institute of Animal Health. This study was approved by the Animal Welfare Committee of the Faculty of Veterinary Medicine University of Nigeria, Nsukka. They were housed in standard animal house, fed standard dog food. They had access to water ad libitum and the surgery was conducted under strict aseptic conditions.

Animals used

Twelve Nigerian indigenous dogs (8-12 months old) were used for the study. They were vaccinated against rabies, parvovirus enteritis, hepatitis, canine distemper and kept in Department of Vet Surgery small animal house. The dogs were fed the same type of food throughout the period of study. Physical and clinical examinations were carried out on these animals to ascertain their health status prior to surgery. These dogs were randomly assigned to four groups of 3 animals each as follows:

Platelet rich plasma-treated group (PRPG); Cancellous bone graft treated-group (CBG), Platelet rich plasma/Cancellous bone graft treated-group (PRP/CBG) and the Normal saline treated-control group- (CG).

Preoperative preparation

Each of the dogs was sedated using 2% xylazine HCl (Indian imunobiologicals) at the dose 0.2 mg/kg body weight intramuscularly as a premedicant followed by intravenous administration of pentobarbitone sodium (6%) (Kyron South Africa) at the dose of 25 mg/kg body weight**.**

The left fore-limbs of these animals were generously shaved from the elbow to the distal 1/3 of the ulna. This area was aseptically scrubbed and draped prior to surgery.

Surgical technique:

Each dog was placed in dorsal recumbency and the left forelimb pulled forward to expose the caudal aspect of the antebrachium. A linear incision was then made starting just below the tip of the olecranon and extended to the proximal third of the shaft of the ulna. The fascia was dissected to reach the diaphysis of the ulna. The deep antebrachial fascia was incised between the extensor carpi ulnaris to permit retraction of the muscles laterally and medially. The dissection was continued around the cranial aspect of the bone using Hohmann retractors to maintain retraction. On getting to the ulna a partial ostectomy of about 3mm was done using osteotome. The defects in each group were then treated before wound closure as follows:

Platelet rich plasma treated group (PRPG):

Dogs in this group (PRPG) were treated with autologous PRP prepared with Plateltex^{R} prep and Plateltex^{R} Act following manufacturer's instructions. After the preparation, the autologous PRP was gently collected with thumb forceps and applied onto the defect of the same dog. The wound was closed by suturing the deep fascia in a simple continuous pattern with size 2-0 chromic catgut. The subcuticular stitches were also placed in a simple continuous pattern. The skin incision was closed with simple interrupted sutures with silk.

Cancellous bone graft treated group (CBG):

The dogs in this group were treated by applying an autologously collected cancellous bone grafts from the proximomedial aspect of the tibia of the dog. Collection of the bone grafts was done under anaesthesia as follows; the proximomedial region of the contralateral tibia was generously shaved with razor blade and scrubbed with gauze impregnated with hibitane hydrochloride. The dog was then positioned in lateral recumbency with the medial aspect of the donor tibia up. The animal was then properly draped ready for surgery. A skin incision was then made at the

medial aspect of the proximal tibia to expose the proximal metaphysis. Cancellous bone graft was then collected using manual bone drill to enter the medullary canal of the metaphyseal region of the bone. Upon removal of the drill bit, a 2-0 bone curette was used in the fashion and motion of an ice cream scoop to collect enough graft to fill the defect. The donor site was then closed routinely. The graft was immediately transferred to the defect on the ulna. The muscles and the skin were then closed with size 2/0 chromic catgut and silk respectively.

PRP/CBG:

The bone defects here were treated by applying both autologous PRP (prepared with Plateltex Prep and Act kits of Czech Republic) and autologous cancellous bone graft onto the defects before closure.

CG:

The dogs in the dogs in this group were treated by applying normal saline on the defects before closure.

Histological study

At week 10, the 3 dogs in each group were subjected to euthanisia and the affected ulna bones dissected out for histological analysis of the callus formed at the bone defects. The bones were fixed in 10% formalin for 15 days. Subsequently bones were decalcified in Goodling and Stewart's fluid containing formic acid 15 ml, formalin 5 ml and distilled water 80 ml solution

and it was stirred daily and changed once in three days. The sections were checked regularly for the status of decalcification. They were considered as completely decalcified when sections became flexible, transparent and easily penetrable by pin. The decalcified tissues were processed in a routine manner and 5 μm sections were cut and stained with Haematoxylin and Eosin.

The level of maturation of the callus was semi-quantitatively evaluated and scored by considering the following histologic indices as modified from Nilsson *et al*. (1986). The average score for the 2 dogs in each group was determined. The mean scores ± sem for all the indices considered were compared.

Indices	**Scores**
Fibrocartillagenous zone still present	0
Presence of calcified cartilage	1
Endochondral ossification	2
Presence of woven bone	3
Presence of marrow cavity	4
Active periosteum	5
Evidence of remodeling	6
Lamellar nature of secondary callus	7

Results

The PRP/CBG defects showed advanced stages of healing with greater amount of lamellar bone and less amount of fibrous tissues compared to the other groups. The PRP defects exhibited remarkable histologic indices. The mean histologic index of the PRP/CBG (2.81±0.70) and that of the PRP (2.69±0.66) however did not vary significantly ($p > 0.05$) from each other but they varied significantly ($p < 0.05$) from those of the dogs in the CBG (1.69±0.53) and CG (0.69±0.31), (Table 1). In the PRP/CBG defects, there was evidence of replacement of woven bone by a more mature lamellar bone (fig.1).There was also cessation of periosteal activities in 66.7% of the defects (fig.1). Approximately 67% of the PRP defects also demonstrated a more matured secondary callus than those of the CBG and CG defects. These secondary calluses were gradually being remodeled and arranged in lamellar nature. At week ten, the CBG (fig.3) and CG (figs.4) defects were still undergoing endochondral ossification with fibrocartilaginous zones of intense cellular activities unlike the PRP/CBG defects.

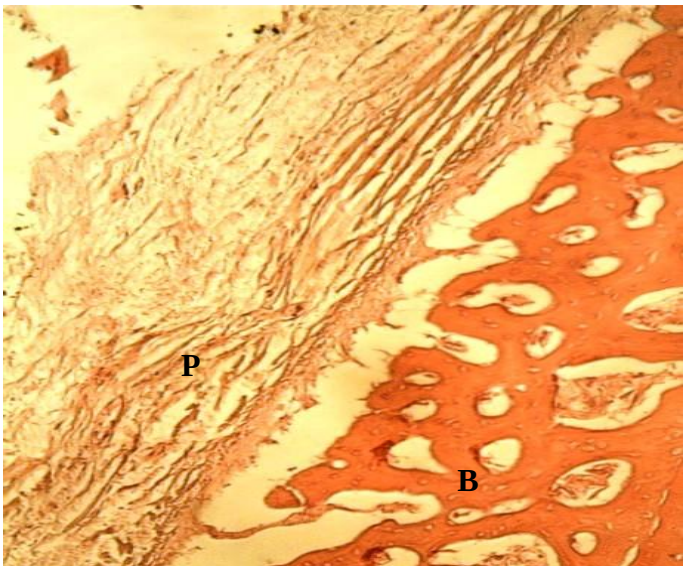

Figure 1: Photomicrograph of external callus of one of the **PRP/CBG** defects at week 10 post treatment, showing replacement of the woven bone by a more mature lamellar bone (B) covered by a normal periosteum (P). Note the cessation of periosteal activity at this point (H & E x 100)

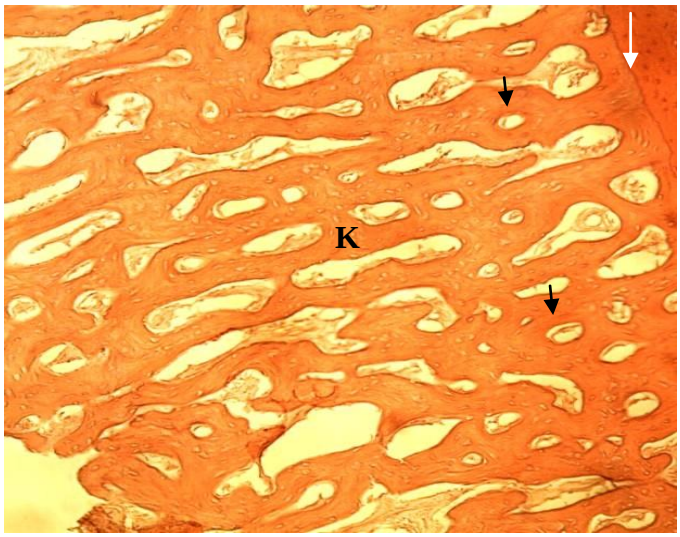

Figure 2: Microscopic view of the callus of one of the **PRPG** defects at week 10 post treatment showing a mature secondary callus (K) attached to the periosteal surface of cortical bone (white arrow) in bone repair. Note the lamellar nature of the secondary callus (black arrows) (H & E x 100)

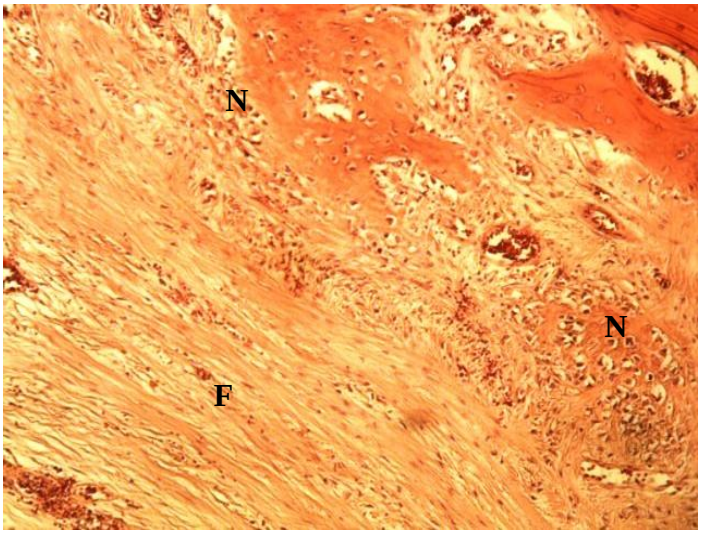

Figure 3 : A photomicrograph of one of the **CBG** defects at week 10,post treatment showing active periosterum characterized by intense fibroplasia (F) and multifocal areas of osteogenic activity (N) (H & E x 100)

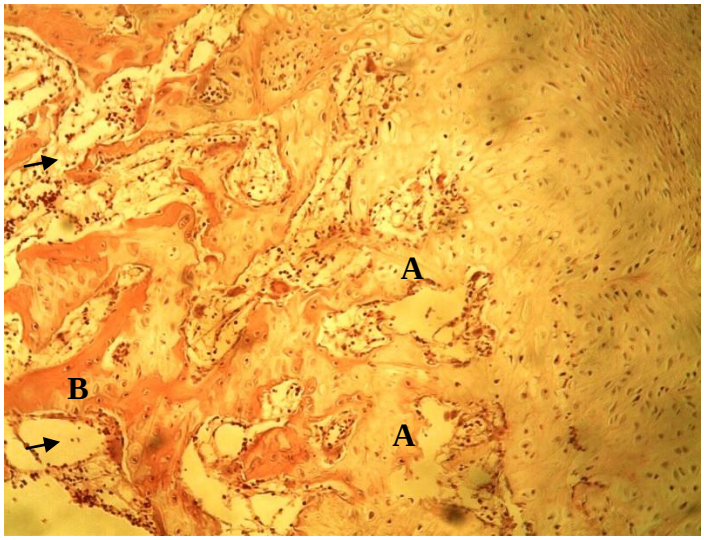

Figure 4: A section of primary callus of the defect of one of the control animals at 10 weeks post treatment showing zones of active cell division/osteogenic activity (A) and newly formed woven bone (B) Note the marrow cavities (arrow). (H & E x 100)

TABLE 1: MEAN PERIODIC OPTICAL DENSITY (±SEM) OF THE BONE DEFECTS OF DIFFERENT TREATMENT GROUPS

	PRP	PRP/CBG	CBG	CG
WEEK1	1.50 ± 0.00^{a}	1.46 ± 0.02^{ab}	1.45 ± 0.02^{b}	1.43 ± 0.02^{b}
WEEK6	1.01 ± 0.01^{a}	0.95 ± 0.03^{b}	1.28 ± 0.02^{c}	1.34 ± 0.02^{d}
WEEK8	0.91 ± 0.03^{a}	0.78 ± 0.02^{b}	0.89 ± 0.01^{a}	1.19 ± 0.02^{c}

a, b, c=mean in same row with different superscript differ significantly ($p < 0.05$)

Discussion

The histological section of the defects showed a more matured cancellous bone in the PRP/CBG treated group (fig 1) unlike in the control group where mainly primary callus characterized by fibrocartilageneous zone of intense cellular proliferation (fig 4) with minimal deposition of woven or cancellous bones were observed (fig 4) suggesting that osteogenesis in the control group had not advanced much compared to the other groups. This could probably be due to the fact that the PRP applications in addition to enhancing bone proliferation in the PRP and PRP/CBG groups also significantly improved the handling of the particulate grafts at the site of the defects in the PRP/CBG group and hence faster rate of graft incorporation and mineralization of the osteoids (Jakse *et al.*, 2003). The more advanced histologic presentation of the PRP/CBG defects could also be ascribed to the fact that the microenvironments of the PRPG/CBG defects in addition to being enriched with multiple exogeneous growth factors also had a good source of osteoprogenitor cells, osteoinductive and osteoconductive scaffold for bone proliferation from the autologous PRP and the cancellous bone graft applied (Everts *et al*., 2006). The control group exhibited juvenile histologic features because the biochemical environment of the defects in this group was not enriched with as much bioactive material as the PRP, PRP/CBG and the CBG treated defects. Normally, the repair of musculoskeletal tissues generally starts with the formation of blood clots and degranulation of platelets, which releases growth factors and cytokines at the sites. This microenvironment results in chemotaxis of inflammatory cells as well as the activation and proliferation of local progenitor cells (Tabata, 2003). Enriching this microenvironment with exogeneous PRP and PRP/CBG could have contributed immensely to the greater osteogenic activities and enhanced healing of the defects seen in the PRP and PRP/CBG groups.

Conclusions

In summary, we found that the use of autologous platelet rich plasma and cancellous bone graft either in combination or singly was efficacious for the treatment of caudolateral ulna defects in dogs, but the performance was optimal when they were applied concurrently due to the reasons outlined above.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Nnaji, Kene, Ogbanya and Chah participated in the histopathological evaluation, literature review, drafting of the manuscript, the final histopathological diagnosis, designed and carried out all the experiments, performed the statistical analysis. Okpe was the principal investigator of the laboratory in which the research was performed and contributed to the interpretation of the data and writing of the manuscript. Udegbunam edited the manuscript and made required changes. All authors have read and approved the final manuscript.

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