Evaluation of single and multiple use of bone marrow aspirate in management of tibia fractures of Nigerian indigenous dogs

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\textbf{ABSTRACT}

Osteogenic potential of autologous bone marrow aspirates was evaluated on transverse tibia fractures of 12 Nigeria indigenous dogs presented to University of Nigeria Veterinary Teaching Hospital, Nsukka (UNVTH). They were assigned into four groups: single application group (Group1; \(n = 3\)), double application group (Group 2; \(n = 3\)), triple application group (Group 2; \(n = 3\)) and the control group (Group 3; \(n = 3\)). The fractures were immobilized with Steinmann intramedullary pins in a normograde manner. The patients were then treated as follows: Group 1: with autologous bone marrow aspirate on the day of surgery; Group 2: with autologous bone marrow aspirate on days 0 and 14 post surgery; Group 3: with autologous bone marrow aspirate on days 0, 14 and 28 post surgery; Group 4: 1 ml normal saline was applied on the fracture site. Post-treatment radiographs of the fractures were taken on weeks 1, 2, 4, 7 and 10. The rate of healing was compared using radiographic union scale for tibia fractures. The results showed a significant difference (\(p < .05\)) in the healing of the treated groups and the control at week 7 post surgery. There was however no significant (\(p > .05\)) difference in the healing of the fractures with single and the multiple applications.

\textbf{Introduction}

Successful bone formation is the key to fracture healing. Previous studies on fracture healing and the repair of segmental bone defects focused on bone matrix substitutes (Grundel et al. 1991; Delloye et al. 1992; Gogolewski et al. 2000). However, these substitute matrices did not perform as well as autograft for several reasons, including histochemical responses by the host tissue and a dearth of living cells. Recently, the osteogenic potential of concurrent application of autologous cancellous bone grafts and autologous platelet-rich plasma on experimentally induced ulna defects of dogs has been elucidated (Nnaji et al. 2015; Nnaji and Kene 2015). Also successful bone regeneration has been achieved using transplantation of mesenchymal stem cells alone or in combination with other biomaterials.

In spite of these, patients in orthopaedic hospitals are still battling with the problem of bone healing. This, to a large extent, calls for more researches on adjunctive means of facilitating bone healing. The use of bone marrow aspirates is one of such means that are being investigated by orthopedists. In 2013, Kassem successfully managed 19 delayed union fractures of human patients using percutaneous autologous bone marrow injections. Kitoh et al. (2004) reported an encouraging result of local injection of both culture expanded bone marrow aspirates and platelet-rich plasma to achondroplasia patients. But, culture expansion technique requires additional visit to the clinic, special equipment and additional training to harvest the stem cells, which may not be feasible in most under developed countries of the world, including Nigeria.

Because bone marrow is known to contain osteogenic progenitors, its implantation was perceived to have the potential to lead to effective bone regeneration in man. Stem cells participate in bone regeneration in different ways. They directly differentiate into tissue-specific cells and thus substitute damaged or lost cells. They indirectly influence tissue regeneration by secretion of soluble factors, thereby promoting vascularization, cell proliferation, differentiation within the tissue and modulation of inflammatory processes (Kalia 1999).

For some times now, researches on the use of bone marrow aspirate to reduce the incidence of delayed bone healing have gained major recognition. However, most of the previous researchers had always concentrated on single percutaneous administration of the aspirate without repetition. This left us with the dearth of knowledge on the outcomes of multiple application of bone marrow aspirate with time as a means of enhancing osteogenesis in bone defects. Secondly, there is paucity of information on the osteogenic effects of bone marrow aspirates on tibial fractures of Nigerian indigenous dogs.

This study was specifically carried out to evaluate the osteogenic effects of single, double and triple percutaneous bone marrow administration on tibial fractures of Nigerian indigenous dogs.

\textbf{Materials and methods}

\textbf{Ethical approval}

All the study dogs were privately owned, and their owners signed informed consent. The study protocol was approved...
by the committee on animal welfare of the Faculty of Veterinary Medicine, University of Nigeria, Nsukka. Date of approval was 5th January, 2015.

**Animals**

Twelve Nigerian indigenous dogs (NID) aged 8 months to 1 year were used for the study. They included those patients that were presented between February 2015 and December 2015 for treatment in UNVTH following transverse fractures of their tibias. (Exclusion criteria included presence of bony deformity, concurrent medical illness, other fractures and massive soft tissue compromise). These patients were equitably and randomly assigned into four groups of three dogs each as follows:

- **Group 1** ($n = 3$): (Injected with BMAC once; day 0 post fixation)
- **Group 2** ($n = 3$): (Injected with BMAC twice; days 0 and 14 post fixation)
- **Group 3** ($n = 3$): (Injected with BMAC trice; days 0, 14 and 28 post fixation)
- **Group 4** ($n = 3$): (Injected with normal saline post fixation).

They were kept in separate kennels and feed with the same type of food. Water was provided to them daily.

**Preoperative procedure/anaesthesia**

Prior to surgery, each patient was stabilized by infusion of appropriate fluids. The haemogram and the serum biochemistry of each patient were evaluated and stabilized to their normal values before the surgeries. Food was withheld for 12 h and basic parameters such as body weight, rectal temperature, heart rate, pulse rate and respiratory rate were checked. A pre-anaesthetic, xylazine (1.0 mg/kg) was administered intramuscularly, and upon sedation, a generous shaving was done around the fracture sites. The areas were aseptically scrubbed and covered with gauze soaked with povidone iodine. The donor sites were also prepared aseptically. Anaesthesia was induced and maintained with ketamine hydrochloride (10 mg/kg body weight intramuscularly).

**Reduction and fixation of the fractures**

Steinmann intramedullary pin was introduced alongside the medial border of the straight patellar ligament through a key-hole incision with the stifle held fixed. It entered the bone at the base of the tibial crest, cranial to the intermeniscal ligament. The insertion of the pin was done with Jacob’s chuck making sure that the hock joints were not entered.

**Collection of bone marrow aspirates**

The procedure was performed under general anaesthesia to avoid stress or discomfort to the dogs. Each dog was placed on lateral recumbency with the donor forelimb up. The elbow was rotated medially and the humerus pushed cranially to expose the shoulder. A stab incision was made between the greater tubercle and the humeral head following strict asepsis. Sternal puncture needle was slowly twisted clockwise and counter-clockwise to seat into the cortical bone of the humerus. The needle was adjusted with a screw driver-like motion until it sat well in the marrow cavity. A 10 ml syringe coated with an anticoagulant (ACD) was firmly fitted with 17 gauge needle and inserted into the marrow. The plunger was rapidly pulled back to 8 ml pressure to aspirate 2 ml of bone marrow in the syringe. This was then transferred into sterile test tubes and centrifuged at 3200 rpm for 15 min.

**Application of the bone marrow aspirate to the fractures**

- **Group 1** (single application of bone marrow aspirate): The bone marrow aspirate was administered on the fracture sites once at the time of surgery.
- **Group 2** (double application of bone marrow aspirate): The bone marrow aspirate was applied on the fracture at the time of surgery and percutanously two weeks later.
- **Group 3** (triple application of bone marrow aspirate): The bone marrow aspirate was administered on the fracture at the time of surgery and percutanously at days 14 and 28 post-operation.
- **Group 4/Control:** There was no application of bone marrow aspirate on this group which serves as the control but rather normal saline was applied at the time of surgery.

**Post-operative management/evaluation**

**Medications**

- Procain penicillin 20,000 iu/kg intramuscularly for 5 days
- Streptomycin sulphate 10 mg/kg intramuscularly for 5 days
- Paracetamol (10 mg/kg body weight) given for 3 days for the management. The skin stitches were removed at day 10 post-operation in all the patients.

**Radiologic evaluations**

Radiographs of the fractures were taken at weeks 1, 2, 4, 7 and 10 post-treatments. The rate of healing was semi-quantitatively evaluated from the lateral and antero-posterior radiographs taken at week 7 using the radiographic union scale for tibia (RUST) as described by Kooistra et al. (2010) and Whelan et al. (2010).

The RUST scoring system was based on the assessment of fracture healing at each of the four cortices (i.e. medial and lateral cortices on the antero-posterior radiograph; anterior and posterior cortices on the lateral radiograph).

Each cortex received a score of 1 point, if there was presence of fracture line with no callus; 2 points, if there was callus present but a fracture line was still visible; and 3 points, if there was bridging callus with no evidence of a fracture line. The individual cortical scores were added to give a total score of 4, which is the minimum score indicating that the fracture had definitely not healed and 12 being the maximum score.
indicating that the fracture was definitely healed (Kooistra et al. 2010). Radiographic fracture union was defined as when bony callus was evident on at least three cortices in standard antero-posterior and lateral views and with RUST scores $\geq 7$.

| Callus     | Fracture line | Score per cortex |
|------------|---------------|------------------|
| Absent     | Visible       | 1                |
| Present    | Visible       | 2                |
| Present    | Invisible     | 3                |

Plate 1. Radiograph of one of the fractured tibia in group 1 before immobilization at week. The arrow shows the fracture.

Plate 2. Radiograph of one of the fractured tibia in group 1 at week 2. The arrow shows the fracture.

Plate 3. Radiograph of one of the fractures in group 1 at week 4. The fracture lines were still clearly visible but there was evidence of periosteal and endosteal callus proliferation in all the animals at this stage.

Plate 4. Radiograph of one of the fractures in group 2 at week 4. The fracture lines were faintly visible at this stage in all the patients being bridged with callus of soft tissue density.
The functional limb usage of the patients was also evaluated in terms of weight bearing on the affected limbs and compared subjectively (Table 1).

The individual rust scores of all the animals in each group were recorded and analysed using One-Way Analysis of Variance (ANOVA). Variant means were separated using Duncan’s Multiple Range Test. Significant level was accepted at probability (p < 0.05) (Plates 1–6).

**Results**

There was a gradual progression from non-weight bearing lameness to full weight bearing among the treated groups. At week 10, 100% of all the animals that had multiple applications exhibited total weight bearing on the affected limbs, unlike the control where we had 33.3%. For radiographic union, at week 7, 100% of the treated groups had union while the control had 33.3% (Plates 7–10).

The treated groups (1, 2 and 3) showed significantly (p < 0.05) higher mean RUST scores (11.00 ± 0.58a), (11.69 ± 0.38a) and (11.69 ± 0.38a), respectively at week 7 than the control fractures (08.67 ± 0.33b) (Table 2).

**Discussion**

These results suggest that bone marrow aspirate had a positive osteogenic activity by contributing to the superior osteogenesis seen in groups 1, 2 and 3 fractures.
This finding could be attributed to the osteogenic activities of the pleuripotent mesenchymal stem cells present in the aspirates concentrates. Sequestration of the aspirates provided higher concentration of nucleated cells and bone spicules per millilitre of the aspirates at the fracture sites and this was of advantage for optimal osteogenesis (McDaniel et al. 2017). These mesenchymal stem cells in the marrow aspirates under optimal mechanical, environmental and adequate supply of oxygen differentiate freely into osteoblasts, which are the bone forming cells. Secondly bone marrow aspirates contain some minute bone spicules, which although small may act as a scaffold for osteoid deposition (Brighton and Hunt 1991). Fracture healing as a biological process is influenced by many mechanical, biochemical and cellular mechanisms of which many cells may be involved (osteoprogenitors, osteoblasts, chondroblasts, osteocytes red blood cells white blood cells) (Nnaji et al. 2015). Many bioactive substances have been proved to enhance osteogenesis due to their inherent ability to differentiate into bone forming cells. Basically, the pleuripotent mesenchymal stem cells in the bone marrow might have participated in bone regeneration at the fracture sites by; (1) directly differentiating into tissue-specific cells and thus substituted the damaged or lost cells, (2) indirectly influencing tissue and bone regeneration by secretion of soluble factors such as growth factors and salts like calcium, phosphorus, potassium, hydroxyapatite salts and (3) by enhancing better vascularization of the osteoid seams and modulating the inflammatory processes (Kalia 1999). Although we did not present the number of cells that were transplanted at the fracture sites, the cytometry findings showed normal total granulocytes and erythrods.

Plate 9. AP and lateral radiographs of one of the fractures in group 3 at week 7. The four cortices had callus and the fracture lines were invisible in ‘a’ and ‘b’.

Plate 10. AP and lateral radiographs of one of the fractures in group 4 at week 7. Note the visible lines (arrows) on the cortices but with callus formation on the cortices of ‘a’ and ‘b’.

Plate 11. Lateral radiograph of one of the fractures in group 1 at week 10. Fracture gaps were completely filled with mineralized callus of osseous density in all the dogs here.

Plate 12. Lateral radiograph of one of the fractures in group 2 at week 10. Fracture gaps were completely filled with trabeculae bones in all the dogs here.
numbers with mean myeloid to erythroid ratio of 1.5. One good thing associated with the use of bone marrow aspirate is that, unlike bone grafts where resorption proceeds in an unpredictable manner (allografts and xenografts), with possible encapsulations and material residue which may interfere with bone remodelling, there is always no encapsulation and no residues left (Percival and Chas 1919).

Groups 1, 2 and 3 fractures (Plates 10, 12 and 13, respectively) showed greater mineralization of the osteoids, as compared to the control fractures (Plate 14) at week 10 as demonstrated by the sclerotic nature of their bridging callus. There was however no significant difference \( (p > .05) \) between the osteogenic effect of the single and double application (Table 2) and this could be ascribed to the fact that the second application was done at week 2 during which the level of phagocytosis of dead tissues and debris at the fracture sites was high. This was due to the activities of the white blood cells which contributed in healing of bones not only by fighting infections but also by ingesting particulate matters, such as dead cells, tissue debris and old red blood cells between and around the fracture gaps, thus causing the widening of the fracture gaps at week 2. Also many of the weak cells in the marrow aspirates might have been recognized as particulate matters, or as dead cells and therefore were engulfed following the second injection and thus could not differentiate into bone forming cells at that time. With this, it was most probable that there won’t be significant variation in terms of the rust scores (Table 2), and other osteogenic indices between the single (group 1) and double (group 2) applications. These results could also be due to mechanical interference with the already formed primary callus at the fracture sites during the percutaneous injection of the aspirate at week 2.

In a nutshell, bone marrow aspirates contain osteogenic stem cells and osteoinductive factors that are associated with an improved formulation of an inorganic scaffold (an osteoconductive material) which has been shown to be a viable regenerative system (Brighton and Hunt 1991). These would have contributed to the positive osteogenic effects exhibited by the single, double and triple applications of bone marrow aspirates in this study. Connolly et al. (1991) believed that the osteopromotive effect of the bone marrow was linked to two osteogenic proteins it contains: higher catalase and glutathione peroxidase. According to them bone marrow has the ability to facilitate osteogenesis by acting as both osteoprogenitor and osteoinductor supplement with limited amount of available autologous bone. Muschler and Midura (2002) also showed that delivery of connective tissue progenitors at a concentration slightly higher than what is naturally found in a bone marrow resulted in a significant increase in the rate of union, bone volume and mechanical stiffness of fractures.

| Group | Individual rust score | Mean ± SEM |
|-------|-----------------------|------------|
| Group 1 | 11 10 12 | 11.00 ± 0.58* |
| Group 2 | 12 11 12 | 11.67 ± 0.33* |
| Group 3 | 12 12 11 | 11.67 ± 0.33* |
| Group 4 | 9 8 9 | 0.87 ± 0.33* |

Note: a,b = mean different superscript on the same column differ significantly \( (p < .05) \).

Plate 13. Lateral radiograph of one of the fractures in group 3 at week 10. Healing is complete but there was large amount of mineralized periosteal callus of osseous density formed at a level even distal to the fracture sites in 100% of all the patients in this group.

Plate 14. Lateral radiograph of one of the fractures in group 4 (control) at week 10. Some cortices were still faintly visible being filled with callus of soft tissue density (arrow). Osteoid mineralization was relatively poor compared to the experimental groups.
Conclusion

Bone marrow aspirate has demonstrated a positive osteogenic effect on fracture healing of Nigerian indigenous dogs. The treated groups exhibited a more matured osteoid seams in terms of callus proliferation and mineralization (greater sclerosis) when compared to the control where fracture bridging with both endosteal and periosteal callus was not appreciable. This study however could not justify any potential benefit of multiple percutaneous injection of bone marrow aspirate over single injection in enhancing tibia fracture healing of Nigerian indigenous dogs.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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