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Comparative biocompatibility studies of Kirschner wire, allogeneic and canine xenogeneic cadaveric bone tissue implants in rat model

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Abstract

Pre-clinical safety or biocompatibility studies of newer biomaterials intended for clinical use are paramount in medical implant technology. The *in vivo* safety of rat (allogeneic) and dog (xenogeneic) cadaveric bone implants (CBI) in the present study were assessed through histological and haematological profiles. A total of 12 male Albino rats (average weight 120 g) divided into three groups (A, B & C) of four rats each was used. Rat (allogeneic) and dog (xenogeneic) cadaveric bone implants were surgically placed between the lateral and cranial vastus muscles (on the thigh) of the rats in groups B and C, respectively, while Kirschner wire/inert implants were used for group A and served as control. The rats were monitored for 30 days, and peripheral blood profiles, as well as tissue sections at intervals of 14 and 28 days, were harvested, studied and compared. Results showed that the overall performance of all groups of rats was good. There was marked cellular infiltration of myocytes in rats of groups B and C, and cartilage-like (hyaline) cells in group C at 14 and 28-days post-implantation, respectively. No significant alteration existed in the haemogram of all groups of rats except for the increased lymphocytes count observed in group B pre-implantation. In conclusion, canine CBI used in this study possess fairly excellent biocompatibility and osteoinductive potentials in the rat's model. Therefore, having confirmed their relative *in vivo* safety and biocompatibility, further clinical trials involving the use of cadaveric bone tissues as good biomaterial for fabricating bone fixatives should be performed in higher animal models.

Keywords: Biomaterials, Fixation, Fracture management, Orthopedic surgery, Osteoinduction

Introduction

In transplantation medicine, any material that does not produce a severe toxic or immunological response when exposed to living tissues or body fluids is considered biocompatible (Veritas Health,

2021). Biocompatibility entails the ability of any material to perform the following recipient tissue response to a specific situation (Bosco *et al.*, 2012). Studying the biocompatibility of biodegradable implants may be achieved through pre-clinical *in vivo* testing. This is usually done by implanting the biomaterial to be investigated into tissues of test animals (e.g., rat, rabbit, sheep, goat, pig or dog), thereafter the animals are trailed to investigate the desired parameters and to monitor tissue healing (Väänänen, 2009). Thus, animal models formed a key component of most nonclinical implant studies, and the ideal species varies by the specific implant and its intended use (Wancket, 2015). Clinical examination, imaging (radiological, ultrasound, MRI, CT), macroscopic and histological evaluations are considered to be part of pre-clinical *in vivo* tests of implants (Väänänen, 2009).

Generally, an ideal bone substitute for fracture repair should have no risk of immunological rejection (biocompatible) or disease infection, good biodegradation and biomechanics similar to the surrounding bone tissues, and must achieve the incorporation of graft in host bone by gradually being substituted with regenerated bone (Kornberg *et al.*, 1999; Sohn & Oh, 2019). Likewise, it should be structurally similar to real bone.

According to Ambrose & Clanton (2004), the biocompatibility of the biodegradable materials currently in clinical use is already established and has been widely reported in the literature. This makes further *in vivo* studies unnecessary if same material is to be used in creating new implants (Väänänen, 2009). However, for implants either containing new biodegradable materials or in itself being biomaterial which have not been previously studied, their biocompatibility should always be tested pre-clinically (Väänänen, 2009). To the best of our knowledge, the biocompatibilities of the canine xenogeneic and rat allogeneic cadaveric bone tissues used in the present study, despite their availability, have not been previously evaluated or used as a biomaterial in producing bone fixation implants, thus, necessitating the study.

Materials and Methods

Ethical clearance

Ethical approval for the use of animals in this study was granted by Ahmadu Bello University Committee for Animal Use and Care, with approval number: ABUCAUC/2019/26.

Laboratory animals (Albino rats)

Twelve male Albino rats (mean weight ± SEM, of 120 ± 0.00 g) were sourced from Laboratory Animal facility, Department of Veterinary Pharmacology and Toxicology, ABU Zaria. Before the commencement of the study, the rats were acclimatized for a period of 20 days. Water and feed were provided *ad-libitum* throughout the acclimatization and experimental periods.

Processing of cadaveric bone grafts for safety studies

Allogeneic Implant: An adult, male Albino rat (weighing 225g) was humanely sacrificed (euthanized using Inj. Ketamine Hydrochloride; Pauco Ketamine Injection®, Kwality Pharmaceutical Ltd, India, at 50 mg/kg via intracardiac route). The fore and hindquarters were harvested, stripped of muscular and soft tissue attachments with blade leaving behind the long bones, which were then air-dried. Using sharp-blunt scissors, the proximal and distal epiphyses and metaphyses were removed, thus transforming the bone fragments into slender chips of various sizes (Plate I C). The prepared bone chips were immersed in 35% hydrogen peroxide solution (Ugolab Productions Nigeria LTD, No. 31A, Burma Road, Sabon Gari, Kano) for 30 minutes, then thoroughly washed using same solution. They were later rinsed with 10% methanol or denatured alcohol solution (Methylated Spirit, Service Pharmaceutical Co., LTD. Benin City, Edo) and allowed to air dry [(a modification of Autograft tissue processing method, as outlined by Musculoskeletal Transplant Foundation (2009)]. The tissues were autoclaved (at 15 pounces per inch, and 161℃) before grafting.

Xenogeneic Implant: Humeral bone from a fresh dog cadaver (that died of neither infectious disease nor suspected tumors) obtained from Necropsy Unit of the Veterinary Pathology Department, ABU Zaria was used in preparing the xeno-implant. The bone was subjected to same treatment and processing as in allo-implant above, until appropriate sizes were produced (Plate I B).

Study design

The 12 male Albino rats were randomly divided into three groups of four rats each as groups A (control), B, and C. Kirschner orthopaedic wire/inert implant, processed rat allogeneic, and canine xenogeneic cadaveric bone fragments were used for groups A, B, & C respectively. The implants were implanted between the lateral and cranial vastus muscles (on the thigh) of the rats, sutured, and monitored for a period of 30 days for physical evidence of rejection

or otherwise. The peripheral blood profile of the rats was analyzed on day 0 (pre-implantation), day 2, 10 and 30 post-implantations. Tissue sections from a representative rat of each group were studied on days 14 and 28.

Surgical procedure

The rats were anaesthetized using Inj. Ketamine hydrochloride (50mg/ml), (Pauco Ketamine Injection®, Kwality Pharmaceutical Ltd, India) at 100mg/kg, via IM route, after which an aseptic surgical skin incision was made on lateral thigh surface to access the vastus lateralis. The respective sterilized implants (1.65cm length each) were surgically placed and buried deep within the vastus lateralis and biceps femoris muscles. The muscles were sutured over the implant immediately with size 4-0 vicryl (Coated VICRYL®, Polyglactin 910, Suture-Ethicon, Ohio USA) in a continuous, ford-interlocking pattern. Skin incisions were later closed routinely using nylon size 3-0 (Nylon Monofilament®, Agary Pharmaceutical. LTD., Lagos Nig.) in a simple continuous pattern (Plate II & III).

Post-operative care and monitoring

Povidone-iodine ointment (10% WOSAN®) was applied on the surgical wound immediately post-operative and continued every other day until complete gross skin healing. The rats were closely monitored daily for 30 days commencing from the day of implantation. Clinical outcomes (swelling, presence or absence of discharges, skin colour change at the site of the wound) and general healing profile were the indices used to grossly assess the

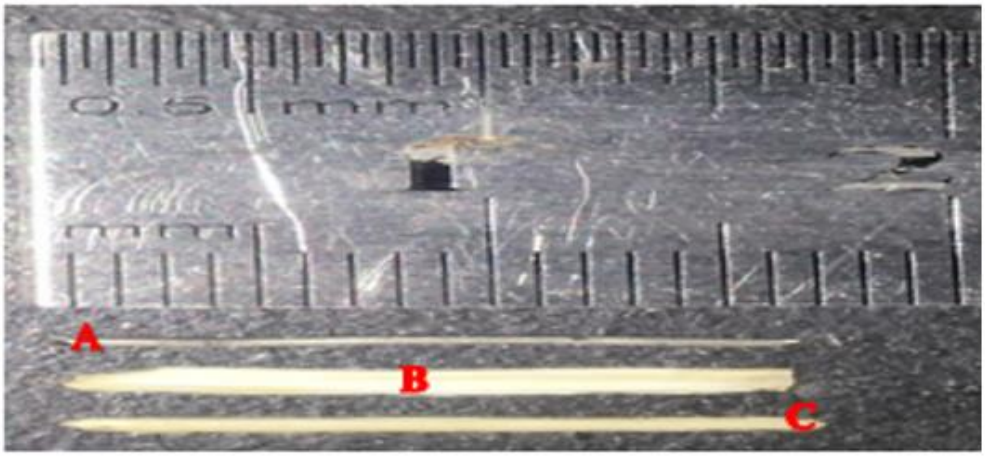

Plate I: A) Kirschner wire/inert implant, B) xenogeneic cadaveric bone implant (from dog), and C) Allogeneic cadaveric bone implant (from rat) used for biocompatibility studies in rats.

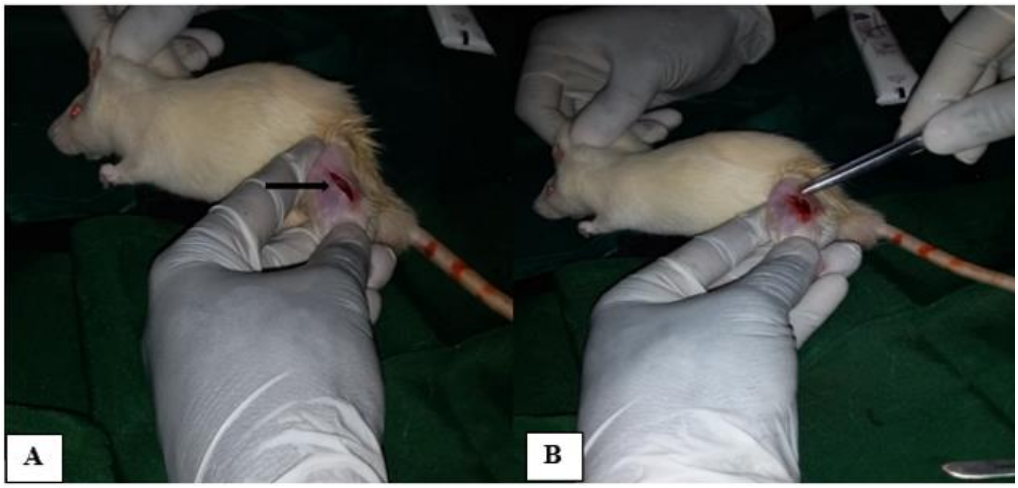

Plate II: A) Cranio-lateral approach through vastus lateralis muscles of a rat (arrowed), and B) proper positioning and grafting of the xenogeneic cadaveric bone implant into the thigh muscles.

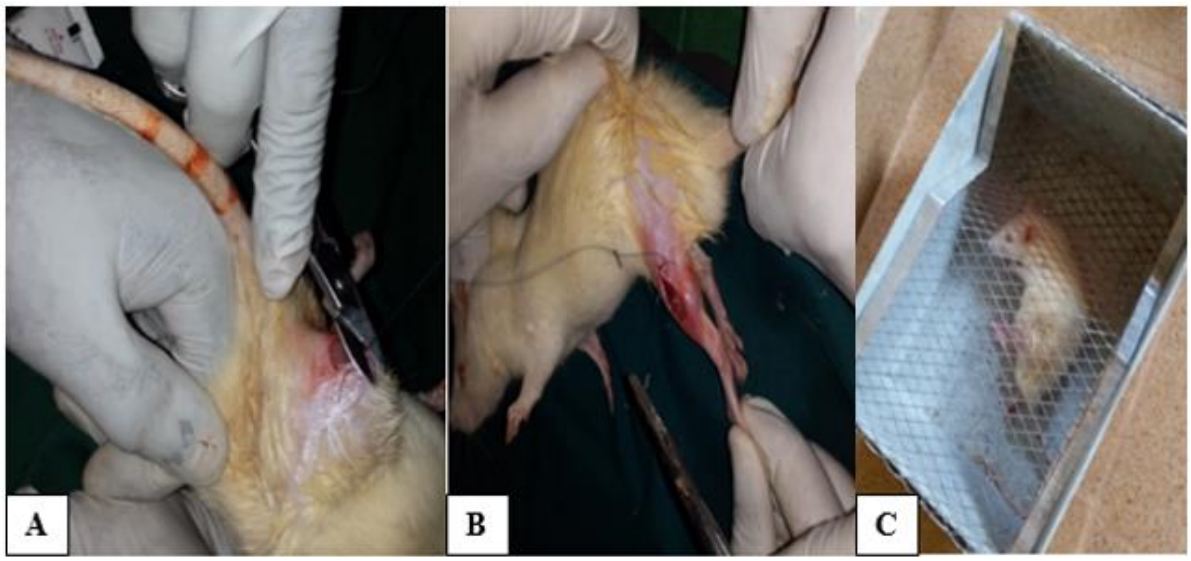

Plate III: A) Closure of vastus lateralis muscle of a rat with size 4-0 vicryl suture post implantation of xenogeneic cadaveric bone implant, B) suturing of the skin and subcutis with size 3-0 Nylon suture, and C) one of the rats recovering from anesthesia in a wire mesh cage.

surgical wounds, vis-a-vis comparing them with those recorded from the control groups.

Samples collected

About 0.5 mL of blood samples were collected from each rat via retro-orbital sinus/vein (Parasuraman *et al.*, 2010), on day 0 (pre-implantation), days 2, 10 and 30 post-implantation using heparinized capillary tubes for haematological analyses as described by Coles (1986). At the 14th and 28th days post-implantation, a representative rat from each group was randomly selected and euthanized. Tissue samples of the thigh (vastus) muscles from the implanted limb were then harvested, fixed in 10% buffered neutral formalin solution (Loba Chemie Pvt. LTD. 107, Wodehouse Road, Mumbai 400005, India.) and labelled for histopathological studies. The fixed muscle tissue was processed based on standard histopathological technique as described by Slaoui & Fiette (2011) and as earlier outlined by Luna (1968). Thereafter, the histoarchitectural slides were studied for evidence of possible immunological reactions.

Data analysis

Two-way analysis of variance (ANOVA) and a mixed repeated measures test was employed for haematological parameters, using Graph Pad Prism® software, Version 5.0 for Windows (Graph Pad Software, San Diego, California, USA, 2009). Total white blood cells, lymphocytes and neutrophils were individually analyzed and compared between the groups. Histopathological tissue slides were presented pictorially and compared.

Result

Rats from all the groups recovered uneventfully from the procedure within the first 7 days of grafting without any noticeable exaggerated responses. On day 11 post-implantation, dyspnea and reduced activity were observed in one out of the four rats in group C (xeno-cadaveric). Likewise, similar signs were observed in a rat from group A (control) at day 15 post-implantation. These signs subsided spontaneously without any intervention 2 to 3 days from the day they were first noticed. On day 30 post-operation, the overall performance of all the rats was adjudged to be good.

The surgical wounds healed by secondary intention with apparent granulation tissue formation in all the groups. These were evident from day 2 through day 14 post-operation. Sutures were removed at day ten postoperatively, and by day 14, the incisional scar had almost disappeared.

Tissue samples collected from randomly chosen representative rats of each group revealed no obvious gross lesions, with all the three implants being identified easily as fibrous encapsulations at the sites of implantation.

The histopathology results showed mild cellular infiltration around the myocytes in the control group (Plate IV A) while the allo and xeno cadaveric implant groups had relatively more exaggerated mononuclear cellular infiltration at day 14 post-implantation (Plates IV B). Interestingly, at 28 days post-implantation, tissues from the allo cadaveric implant rats showed few cellular infiltrations (Plate V A) whereas those in control and xeno cadaveric implant rats demonstrated various degree of mononuclear cell infiltrations, and cartilage-like cells (Plate V B), respectively.

The pre-implantation mean (± SD) packed cell volume (PCV) of groups A, B and C rats were 41 ± 12, 37 ± 6.00 and 43 ± 10, respectively. These values correspondingly dropped two days post-implantation to 30 ± 6.0, 23 ± 14 and 34 ± 0.0. Thereafter, the values were elevated at 30 days post-implantation. However, the differences were not significant (P > 0.05). A similar trend was observed for both total erythrocyte (RBC) count and haemoglobin concentration, and the differences were equally not significant (P > 0.05) (Table 1).

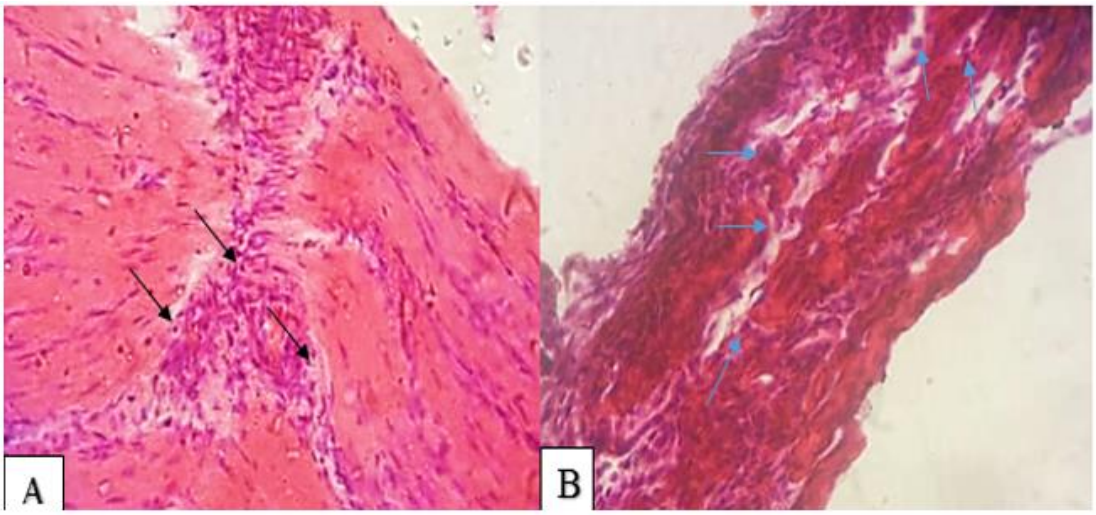

Plate IV: Photomicrograph of tissue sections of rats implanted with Kitschner wire (A), and allo-cadaveric bone implant (B) at day 14 post-grafting. The black arrows showed mild inflammatory cell infiltration, while the blue arrows revealed an exaggerated mononuclear cells infiltration (H & E stain ×100)

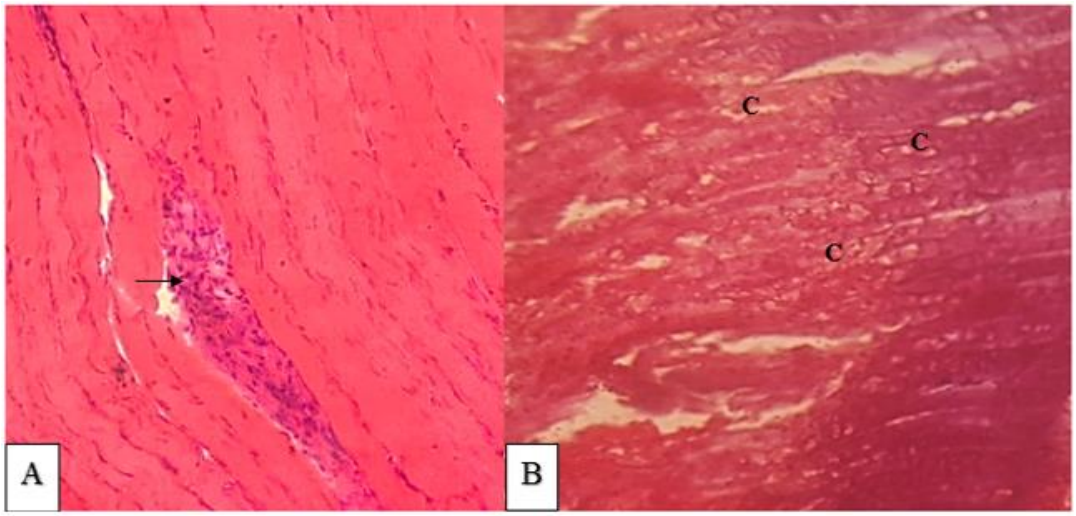

Plate V: A) Photomicrograph of tissue section of rat implanted with allo-cadaveric bone showing normal histoarchitecture with minimal cellular infiltration (black arrow); and B) xeno-cadaveric implanted rat tissue showing myocytes being gradually infiltrated with cartilage-like cells "C" at day 28 post-grafting (H & E stain ×100)

Table 1: Mean (± SD) haematological values of rats grafted with Kirschner wire, *Allogeneic* and *Xenogeneic* cadaveric bone implants at three different observation periods

Haematological Parameter	Day	Groups		
		Group A	Group B	Group C
PCV (%)	0	41 ± 12.00	37 ± 6.00	43 ± 10.00
	2	30 ± 6.00	23 ± 14.00	34 ± 0.00
	30	52.5 ± 25.00	56 ± 8.00	45.5 ± 11.00
Total WBC ($\times10^6$/L)	0	9.9 ± 0.60[a]	19.25 ± 2.3[b]	11.05 ± 0.50[a]
	2	17.1 ± 12.00	12 ± 2.20	9.9 ± 2.60
	30	10.75 ± 0.90	9 ± 1.60	8.2 ± 4.20
Lymphocytes ($\times10^6$/L)	0	6.5 ± 1.28[a]	13.12 ± 2.34[b]	7.57 ± 0.02[ac]
	2	10.82 ± 6.10	8.72 ± 1.96	7.19 ± 1.30
	30	7.3 ± 1.00	5.55 ± 1.26	4.72 ± 2.12
Neutrophils ($\times10^6$/L)	0	3.3 ± 0.88	5.17 ± 0.54	3.26 ± 0.26
	2	5.15 ± 5.42	2.29 ± 0.66	2.49 ± 0.86
	30	2.89 ± 0.62	3.36 ± 0.16	3.37 ± 2.34
Eosinophils ($\times10^6$/L)	0	0 ± 0.00	0 ± 0.00	0 ± 0.00
	2	0.79 ± 0.20[a]	0.89 ± 0.20[a]	0 ± 0.00[c]
	30	0.06 ± 0.12	0 ± 0.00	0 ± 0.00
Band cells ($\times10^6$/L)	0	0 ± 0.00 [a]	0.59 ± 0.46[b]	0.06 ± 0.12[a]
	2	0.35 ± 0.68	0 ± 0.00	0 ± 0.00
	30	0.16 ± 0.12	0 ± 0.00	0 ± 0.00
Monocytes ($\times10^6$/L)	0	0.10 ± 0.20	0.39 ± 0.06	0.17 ± 0.12
	2	0 ± 0.00	0.11 ± 0.22	0.23 ± 0.46
	30	0.33 ± 0.24	0.15 ± 0.30	0.12 ± 0.24
Hgb (g/dL)	0	13.6 ± 4.00	12.3 ± 2.00	14.3 ± 3.40
	2	10 ± 2.00	7.65 ± 4.70	11.3 ± 0.00
	30	17.45 ± 8.30	18.65 ± 2.70	15.15 ± 3.70
RBC ($\times10^{12}$/L)	0	6.95 ± 2.10	6.35 ± 0.50	7.25 ± 1.90
	2	5.25 ± 0.50	4.00 ± 2.00	5.8 ± 0.40
	30	8.9 ± 3.80	9.30 ± 1.40	7.9 ± 2.20
Total protein (g/dL)	0	7 ± 0.40	7.2 ± 0.40	7.4 ± 0.80
	2	7.4 ± 0.80	8.1 ± 1.80	7.1 ± 0.20
	30	7.45 ± 1.10	6.6 ± 1.20	7.25 ± 0.30

Note: PCV = Pack cell volume, WBC = White blood cell, RBC = Red blood cell, Hgb = Haeoglobin concentration, and Values carrying different superscript letters within the same row indicate statistical significance ($P < 0.05$). Blood samples of day 10 (& results) got mixed up and couldn't be retrieved; as such are not presented here

Conversely, the pre-implantation mean (± SD) total leucocyte (WBC) count for group B rats (19.25 ± 2.20) was significantly higher ($P < 0.05$) than those recorded in groups A (9.9 ± 0.60) and C (11.05 ± 0.5) rats. At 2 days post-implantation, the WBC count dropped in the allo- and xeno-cadaveric implant groups while a sharp rise was recorded for group A rats. However, at day 30 post-grafting, all the values among the three groups declined to near pre-implantation figures, and the differences were not significant ($P > 0.05$) (Table 1).

The mean (± SD) lymphocytes count follows exact pattern of the total WBC counts whereas the neutrophils, monocytes, and total plasma proteins were all within similar ranges for the three groups at every stage of the study. While eosinophil count was significantly higher ($P < 0.05$) in the xeno-cadaveric implant group at 2 days post-implantation, but by day 30, the eosinophil values returned to initial pre-implantation range for all the groups (Table 1).

Discussion

Establishing *in vivo* safety and the performance of novel biomaterial substances is very important and can only be investigated by determining such material's potential toxicity or otherwise, which

could result from their contact with tissues and cells of experimental animal models (Sun *et al.*, 2016). The absence of neither local nor systemic exaggerated responses and or inflammation observed in the rats from all the groups may indicate favourable performance (biocompatibility) of the implanted cadaveric tissues, as postulated by Bosco *et al.* (2012). Rats were chosen for this investigation due to their potentiality in biocompatibility studies as the ideal (standard) method in detecting tissue responses whenever a biomaterial is implanted (Sethuraman *et al.*, 2006).

Muscle tissue samples around the site of implantation revealed no obvious gross lesions, with all three implants being identified as just a lump of fibrous tissue encapsulations. These findings, according to Sethuraman *et al.* (2006), are the most normal host defence responses against the foreign material. Subramanian *et al.* (2013) reported that implantation of inert biomaterials and those of known biocompatibility also cause fibrous tissue encapsulation, whereas toxic biomaterials lead to cell death.

The histoarchitecture of muscle tissues from the site of implantation was fairly normal, with varying degrees of mononuclear cell permeation within the period assessed. Infiltration of muscle tissues surrounding an implant material within few weeks of grafting by inflammatory cells (polymorphonuclear leukocytes, macrophages and foreign body giant cells) have been well documented (Peluso *et al.*, 1994; VandeVord *et al.*, 2002). Likewise, the accumulation of hyaline-like cells in the thigh muscle tissues of xeno-cadaveric bone grafted rats at 28 days post-implantation denotes good osteoinductive properties of the tested graft implant as similarly reported by Albrektsson & Johansson (2001). Being a specialized connective tissue, blood is the first to interact with any implanted material when introduced into the body (Paramitha *et al.*, 2015). As part of the body's immune system, leucocytes are specifically transported to the body part that has any foreign substance, infection or inflammation, which serve as an indicator for systemic inflammatory response (Noviana *et al.*, 2012). The immediate upsurge, although not statistically significant in the population of neutrophils two days post-implantation for all the groups, concurs well with the findings of Paramitha *et al.* (2015), who observed that the predominant inflammatory cell types vary in accordance with the age of an injury, and neutrophils are almost always the first to be recruited to injury sites. As the healing progresses, neutrophils are normally replaced by monocytes which are subsequently transformed into macrophages. Also, the absence of eosinophils (Wood, 2017) and basophils (Paramitha *et al.*, 2015; Wood, 2017) at later days and throughout the post-implantation period, respectively, suggests the likely absence of allergic reaction and therefore confirming the high level of biocompatibility of the implants.

The allogeneic and xenogeneic cadaveric bone implants assessed in the current study have good host acceptability with no apparent immune rejection potentials as determined through *in vivo* biocompatibility, clinico-histological studies, as well as peripheral white blood cell profiling. However, the modified chemical treatment and autoclaving of the implants might have affected the bone structural proteins and cells, as well as the immunogenicity of the tissues, thereby contributing to the excellent grafts' acceptance recorded during the in-vivo pre-clinical trial. Therefore, the tested cadaveric implants might likely be good candidates for use as bone fixation biomaterial implants in managing fractures as they have shown to be non-toxic, with no apparent clinical, histological or haematological evidence of incompatibility. Further, *in vivo*, clinical trials in higher animal models should be trialled by direct implantation into the bone. In addition, more elaborate approach of evaluating biocompatibility including immunological assays and immunohistochemical analysis should be employed in further studies. This should be extended beyond 30 days post-implantation to cover any possible long-term reactions.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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