ORIGINAL ARTICLE

Laboratory-Based Anatomical Assessment of the Rabbit Tibia and Evaluation of the Female New Zealand White Rabbit as A Model for Bone Substitute Testing

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ABSTRACT

Objective: To assess the rabbit tibia and to evaluate the female New Zealand White rabbit as an animal model for bone tissue engineering.

Study Design: Laboratory-based experimental study.

Place and Duration of Study: This study was conducted at the Anatomy Department, Army Medical College, Rawalpindi, and the National Institute of Health (NIH), Islamabad, Pakistan. The study duration was from August 2021 to January 2023.

Methods: Twenty New Zealand White rabbits, divided into four groups (5 rabbits in each group), were used and weighed before surgery. After Anesthesia, critical-sized bone defects were created in the right tibiae of each rabbit. Group A was used as control while the rest were filled with the Silicon substituted hydroxyapatite (Si-HA) alone (Group B), Si-HA and Lipoaspirate derived stromal vascular fraction (SVF) (Group C), Si-HA and SVF (modified) (Group D). All the rabbits were weighed again before being euthanized after 6 weeks. After dissecting the tibiae, the length and gross features of each tibia were noted.

Results: After six weeks, an increase in body weight was observed in rabbits of all groups. The maximum increase in weight was 600g observed in rabbit number 3 of Group B, while the minimum was 100g in rabbit number 1 of Group D. Statistically, Group A had the highest average weight gain, but the differences between groups were not statistically significant. After dissection, all tibiae showed normal gross morphological features with the same pattern in length and differences existing within the normal anatomic range. The maximum length of the tibia was 10.5cm, the minimum length was 9.3cm, and the mean length was recorded as 9.9cm.

Conclusion: Standard laboratory diet seems to increase the weight of rabbits in captivity with a reciprocal increase in the dose of anaesthesia as per recommendation.

Keywords: *Adipose Tissue, Bone, Bone Substitutes, Experimental Animal Model, Tibia.*

How to cite this: *Khan MM, Butt SA, Hamid S, Kiani RB, Mahmood M, Anjum K. Laboratory-Based Anatomical Assessment of the Rabbit Tibia and Evaluation of the Female New Zealand White Rabbit as A Model for Bone Substitute Testing. Life and Science. 2026; 7(1): 46-53. doi: http://doi.org/10.37185/LnS.1.1.691*

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Received: Apr 15, 2024; 1st Revision Received: Nov 22, 2024
2nd Revision Received: May 30, 2025; Accepted: Jun 05, 2025

Introduction

For evaluation of new bone substitutes, although in vitro studies with cells, tissues, or organ cultures are conducted, these studies are not adequate to see the material's biocompatibility, mechanical functioning, and safety in humans or animal models.[1]

Most existing preclinical animal studies for the evaluation of biomaterials only consisted of a single-site bone defect with drastic variation in the anatomical locations. Animal models closely represent humans in certain physiological and pathological conditions, and despite this

resemblance as an approximation, the use of animal models acts as a bridge between in vitro and clinical studies by providing valuable information. Compared with in vitro tests, in vivo studies not only allow evaluation of materials under loaded conditions but also for longer time periods in both healthy and diseased states.[2,3] It has been observed that, with time, implanted material degrades and is resorbed, with remnants and debris often found at distant sites, which can only be evaluated in animal models.[4] Regarding bone regeneration, animal models through histologic evaluation provide an in-depth analysis of the material for its osteogenic, osteoinductive, and osteointegration properties along with any abnormalities, if any.[2] Before selecting an animal model for a certain experiment, the research aims and objectives must be clearly defined, as the selected animal should be able to bear physiological and pathological resemblance to humans.[2] Additional factors such as life span and size of the species, vulnerability or resistance to certain diseases or infections, surgical tolerance, and supporting staff should also be taken into consideration.[5,6]

Considering bone tissue engineering research, where the study is primarily based on seeing bone-material interaction and osteogenesis, it is essential to have an in-depth knowledge of anatomy, histology, composition, and bone remodeling properties of the species to be used in the study.[1,7]

Animal handling and care are outlined in different countries' animal protection acts, which share the same objectives, albeit with slight variations. Usually, a local ethical committee is responsible for approving the research protocol. As per these acts, before and during research, the minimum prerequisites, such as housing, lighting, feed, etc., must be met. Ethical issues on animal experimentation emphasize the principles of minimum numbers of animals to be used for research purposes, minimal Pain or distress during experiments, and replacement (if possible) with non-animal alternatives such as in silico models (based on simulations or predictions).[8]

Numerous animal species are used to evaluate biomaterials, each with its own advantages and disadvantages. Considering material implantation for in vivo testing, International standards provided suitability for rabbits, sheep, pigs, dogs, and goats, along with short-term and long-term implantation time frames. Usually, long bones and calvaria are used for the implantation of the material or implant, mostly in the diaphysis, metaphysis, or epiphysis.[9,10]

This study aims to emphasize the use of New Zealand white rabbits as an experimental model in the evaluation of biomaterials, providing a clinically relevant testing ground for further bone tissue engineering efforts.

Methods

The study was conducted at Anatomy Department, Army Medical College, Rawalpindi and National Institute of Health (NIH), Islamabad, Pakistan from August 2021 to January 2023 after taking approval from Ethical Review Committee of Army Medical College, Rawalpindi vide letter no: 9/18/438/ERC/AMC, dated: 14th September 2018. All rabbits were quarantined for 7 days for acclimatization and fed with a standard laboratory diet. Female New Zealand white rabbits (Oryctolagus cuniculus), divided into four groups (5 rabbits per group), with an average age of 10 months, were used for the experimental study. Male rabbits, female rabbits with pregnancy or with any disease, were excluded from the study.[11]

All the rabbits were weighed before the surgical procedure for the calculation of the correct dose of Anesthetic agents and pain killers, and to see the effects six weeks after surgery.[12]

For creating the bone defect, rabbits were anaesthetized and operated upon as before.[13] Briefly, rabbits were sedated using intramuscular injection of Xylazine 5mg/kg (Xylax® 2%, 25mL) and Ketamine 35mg/kg (Ketolar® 50mg/mL) in the left lateral thigh.[14] After disinfection with 10% povidone-iodine, an incision was given on the medial side of the tibia, and a critical-sized bone defect (CSD) measuring 9mm x 6mm x 6mm was created in each tibia with a surgical drill (Escort-III Micromotor, Saeyang Marathon, H20, South Korea).[6] In Group A, designated as control group, defect was kept as such, while the biomaterial alone was placed in defects of Group B. Stromal vascular fraction (SVF) was isolated from adipose tissue enzymatically and non-enzymatically of the right inguinal region of each Rabbit, and was implanted along with the

biomaterial (Silicon substituted Hydroxyapatite) in Groups C and D respectively.[13] Skin was closed with Metallic staples and antibiotics (Inj Enrofloxacin 10mg/kg, Germany) and pain killer (Inj Tramadol, 5mg/kg) given intramuscularly for 5days post operatively. The rabbits were placed in individual pans, and the staples were removed after 10 days. Rabbits were euthanized with Chloroform by inhalation in a closed chamber after 6 weeks. For gross morphological observations, tibiae were dissected.[12,13] All the bones were carefully cleared of connective tissue by not disturbing the defect area, and the tibial length was determined by measuring the distance between the proximal tibial eminences and the distal tibial plafond. Each tibia was measured with a standard ruler to find out any abnormality in the length which might have occurred during the experimental period.[15] (Figure 1).

To test if there are statistically significant differences in mean weight gain among the four groups, Data analysis was carried out by applying one-way ANOVA. Post hoc analysis was performed using Tukey's HSD in the Statistical Package for the Social Sciences (SPSS) version 20. *P*-value of 0.05 or less was taken as significant.

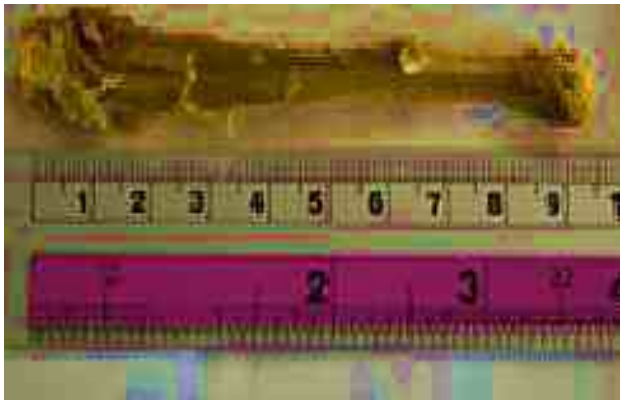

Fig.1: Bone measurement

Results

Preoperatively, the weight of rabbits in all groups varied from 1700g to 2700g, with a mean weight of 2056g. Before sacrificing, all rabbits were weighed again to see change in weight after the experimental period. An increase in body weight was observed in all groups of rabbits. The weight of rabbits varied from 2200g to 3100g, with a mean weight of 2517g. The mean weight increase, group-wise, is shown in Table 1.

In Descriptive statistics, Group A has the highest

Table 1: Initial weight before procedure and weight after six months (in grams)

Group	Animal No	Initial weight Before procedure	Weight after 6 weeks (gms)	Mean weight difference (gms)
Group A	1	1700	2200	129.1
	2	2200	2450	
	3	2000	2600	
	4	2300	2650	
	5	2100	2600	
Group B	1	2100	2250	135.9
	2	2090	2600	
	3	2100	2350	
	4	2500	2850	
	5	1900	2250	
Group C	1	2590	2750	174.7
	2	2280	2450	
	3	2350	2800	
	4	2100	2650	
	5	1900	2350	
Group D	1	2300	2400	141.4
	2	2700	3100	
	3	2100	2600	
	4	1950	2300	
	5	1750	2150	

Table 2: Calculated dosage of anesthetic agents

Groups	Animal no	Inj Xylaxine (mg) (5mg/kg)	Inj Ketamine (mg) (35mg/kg)
Group A	1	8.50	59.50
	2	11	77
	3	10	70
	4	11.50	80.50
	5	10.50	73.50
Group B	1	10.50	73.50
	2	10.4	73.15
	3	10.5	73.50
	4	12.50	87.50
	5	9.50	66.50
Group C	1	12.95	90.65
	2	11.40	79.80
	3	11.75	82.25
	4	10.50	73.50
	5	9.50	66.50
Group D	1	11.5	80.50
	2	13.50	94.5
	3	10.5	73.50
	4	9.75	68.25
	5	8.75	61.25

mean weight gain, while Group C showed the highest variability. In Group comparisons, the differences in average weight gain between groups are not statistically significant at the 5% level. Regarding the induction of anesthesia, a single dose of Inj Xylazine was given to all rabbits, while multiple doses of Injection Ketamine were required for proper sedation of rabbits. (Table 2).

Two rabbits died due to anesthesia, which were replaced to complete the sample size.

Observation of the surgical site after six weeks showed a clearly healed wound without evidence of dehiscence or infection. The bones showed normal gross morphological features. In all the rabbits, the body of the tibia was three-sided proximally and flattened distally. The tibial crest was sharp and thin. Proximally, the interosseous space and popliteal line could be seen in the leg. Also, the lateral and medial condyles, along with the intercondyloid eminence, were also visible. The tibial tuberosity and tibial crest were easily recognizable and can be palpated on external examination. The small medial malleolus, which is an extension of the tibia, was also easily identifiable. There were no signs of infection or bone loss. (Figure 2).

All tibiae showed the same pattern in length, with

Fig.2: Dissected tibia

differences only existing within the normal anatomical range. The maximum length of the tibia was 10.5cm, the minimum length was 9.3cm, while the mean length was recorded as 9.9 cm. (Table 3).

Table 3: Length of bones (cm)

Groups	Animal no	Length (cm)	Mean length (cm)
Group A	1	9.8	9.9
	2	10.3	
	3	9.5	
	4	10.5	
	5	9.5	
Group B	1	10.3	10
	2	10	
	3	9.6	
	4	9.7	
	5	10.4	
Group C	1	9.5	9.8
	2	10.1	
	3	9.9	
	4	9.5	
	5	10.1	
Group D	1	10.4	10
	2	9.3	
	3	10.8	
	4	10.2	
	5	9.6	

Discussion

CSDs have been considered a significant obstacle for orthopaedic surgeons due to bone loss or excision in cases of trauma, blast injuries, and bone tumors, etc. Recent advancements to deal with CSDs are the concept of Bone tissue engineering (BTE), including a combination of natural or synthetic biomaterials, either alone or in combination with implanted stem cells or cytokines etc.[16]

This study was conducted to assess the rabbit Tibia anatomically and also to evaluate the female New Zealand White rabbit as a model for bone substitute testing. Two rabbits died during anesthesia, and they were replaced to complete the sample size. Afterwards, all the rabbits survived to the end of the study, and no wound infections or dehiscence were observed in any of the animals. Rats and mice although are one the most commonly used animal species in research, yet due to their small size and differences with human bones, are not considered for multiple implant evaluation. On the other hand, utilization of large animals such as dogs, goats, and pigs, etc., is limited owing to their high prices and housing.[6,17,18] Differences in bone structure and remodeling exist between human and animal models. For instance, in rats during remodeling, lamellar bone is not formed. Similarly, rodents have a high bone turnover rate, while sheep, canines, pigs, and primates appear to have bone formation closer to humans.[19] Although differences exist between the rabbit and human bones histologically and in terms of skeletal growth and bone turnover, rabbits are still

preferred as an animal model for bone tissue engineering and evaluation of biomaterials, owing to the fact that their bone quality is similar to human bone (Type II bone).[19,20] Rabbits have been used in about 35% of musculoskeletal-related research studies due to similarities to human bones in bone mineral density and fracture toughness. Rabbits are small in size, with the added advantage of limiting the sample size, as not only can multiple implants be placed, but they can also be their own controls.[21] We recommend Female New Zealand white rabbits for such studies due to the similarity of their bony tissue to humans, but also they are easy to manipulate and maintain in captivity as they are not aggressive.

Rabbits have been used by Javaid S et al. for comparative biochemical analysis of platelet-rich plasma-chitosan and platelet-rich fibrin-chitosan for treating tibial bone defects.[22] Feng C et al. used a three-Dimensional Printed (3D) scaffold prepared from akermanite (AKT) and β-tricalcium phosphate (TCP), implanted in the distal femoral condyle of rabbits.[23] Similarly, Lei H. et al. used Icariin-loaded 3D-printed reconstruction rods to treat necrotic femoral heads in rabbits.[24] In our studies, we found that the tibia was easy to operate on and the Tibialis anterior muscle can easily cover the defect after the biomaterial is placed in the defect. In addition, the medial surface of the proximal epiphysis of the rabbit tibia is wide and slightly convex with no muscle insertion, making it easy to create defects of varying size. The rabbit tibia is larger and, being subcutaneous, is less technically demanding during surgical procedures.[25]

While there is some information in the literature on the length of the tibia in rabbits, we found a paucity of information in this regard in local settings, as environmental and dietary factors may introduce some variation. In this regard, Lee KM et al. in their study, measured the length of rabbit tibia in 12 rabbits, in which they found the mean tibial length to be 97.2mm (9.72cm).[15] Similarly, in studies conducted by Crum JA et al. the mean length was 107.1 ± 2.3 mm (10.7cm) in 7 rabbits.[26] Considering the gross anatomical features of the tibia, in our study, the body of the tibia was expanded proximally and flattened distally. Different tibial features, such as tibial tuberosity, tibial crest, interosseous space, popliteal line, and lateral condyles, along with intercondyloid eminence, were easily identifiable. In a comparative study between rabbits and cats by El-Ghazali HM et al., the length of the tibiae of rabbits was about 10.040 ± 1.0102 cm in a sample of 10 rabbits, and the tibiae expressed the same gross features.[27] The findings of the above studies corroborate our findings, and it seems that the features and length of the tibia in different settings remain more or less the same in different settings using the same breed of experimental animals. Additionally, the sample size in our study was larger (N=20) as compared to other studies as well, and can be considered adequate for studies of shorter duration.

The body weight of the control and experimental rabbits was documented before implantation of the biomaterial, as well as 6 weeks after the experimental duration, before euthanizing, as it is considered an indicator of recovery.[14] Increase in weight of all rabbits suggested that all procedures went uneventful without infection or complication. None of the rabbits showed weight loss; in fact, all showed a moderate increase in weight with the standard laboratory diet, as shown in Table 3. The weight of the animal is also documented to calculate the exact dose of medication, such as anesthetics and antibiotics. It can be seen from our results that the dose of medication is directly proportional to the dose of anesthetic agent given e.g. rabbit weighing 2700 g needed inj Xylazine in a dose of 13.50mg and Inj Ketamine in a dose of 94.5mg while rabbit weighing 1700Gm needed inj Xylazine in a dose of 8.50 mg and Inj Ketamine in a dose of 59.5mg for complete induction. This further highlights the weighing of the animal model before any surgical procedure is carried out.

Considering the ethical aspects, legislation imposed by different Governments restricts any harm, injury or suffering to animals during research by the researchers.[2,3] Moral views and social acceptance also play an important role in the selection of the animal for research purposes. In this regard, rabbit is not only socially acceptable by society but also recommended by scientists for musculoskeletal studies.

There are fewer veterinarians specialized in rabbits

in our setup, with less available literature on their specific care. Establishing and developing rabbit bio resource centers would facilitate the acquisition of rabbits and the sharing of valuable information among researchers, thereby reducing costs and space requirements. Future research should focus on leveraging novel genome-editing technologies, such as CRISPR/Cas9, to develop new models for a broader spectrum of human diseases.

Conclusion

It can be concluded from our findings that the tibia is easy to operate, is less technically demanding in surgical procedures, and it's easy to create defects of varying size on the medial surface of the proximal epiphysis. Female New Zealand white rabbit (Oryctolagus cuniculus) can be considered as an appropriate experimental animal model for BTE due to its resilience, and the standard laboratory diet seems to have the propensity of increasing the weight of rabbits in captivity.

Acknowledgement: None

Conflict of Interest: The authors declare no conflict of interest

Grant Support and Financial Disclosure: None

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Author Contributions

MMK: Conception and design of the work
SAB: Manuscript writing for methodology design and investigation
SH: Revising, editing, and supervising for intellectual content
RBK: Validation of data, interpretation, and write-up of results
NM: Data acquisition, curation, and statistical analysis
KA: Writing the original draft, proofreading, and approval for final submission