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Research Article

Evaluation of bone remodeling: robustness and reproducibility of a murine model of mandibular bone lesions

NKOLO TOLO FD[1*], NKECK JR[2], ATANWO DONGMO LN[1], EKO M[3], TANETCHOP MAGOUO N[1], OBONO EKAMENA MJ[1], NJIKI BIKOI JAKY[4]

[1]Department of Oral Surgery, Faculty of Medicine and Biomedical Sciences of the University of Yaounde I, Cameroon

[2]Department of Internal Medicine, Faculty of Medicine and Biomedical Sciences, University of Yaounde I, Cameroon

[3]Department of Biochemistry, Faculty of Medicine and Biomedical Sciences, University of Yaounde I, Cameroon

[4]Department of Microbiology, Faculty of Science, University of Yaounde I, Cameroon

**Corresponding author: nkolo.tolo@fmsb-uy1.cm, +237694144681*

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ABSTRACT

A detailed understanding of the kinetics of mandibular bone consolidation requires robust experimental models. This study assesses the robustness and reproducibility of a Wistar rat model of mandibular bone lesions. The study was conducted on 24 Wistar rats, divided into three experimental groups of 08 rats. The first group served as control. The second group underwent extraction of the lower central incisor with injury to the underlying alveolar bone. The third group underwent drill holes in the mandibular symphysis. The rats were sedated by intraperitoneal injection of ketamine and diazepam. Tooth extraction was performed with an incisor forceps, and drill holes with a micromotor and steel drill, under saline irrigation. All animals received post-operative antibiotic and analgesic treatment. Alkaline phosphatase (ALP) activity was significantly increased in females (289.33 to 437 IU/L, $p<0.01$) and males (56.75 to 303.67 IU/L, $p<0.01$), indicating a more intense and prolonged osteogenic response with drilling than with tooth extraction. In females, the drill hole significantly decreased calcium to 55.5 mg/L ($p<0.05$), whereas in males, tooth extraction significantly increased it to 24.3 mg/L ($p<0.05$) and drilling to 128.25 mg/L ($p<0.05$), suggesting a variable bone remodeling response according to sex and lesion type. In females, phosphorus increased significantly from week 1 in the drill hole group ($p<0.05$), reaching a mean value of around 100 mg/L, while in males, the progressive increase in phosphorus in the same group occurred between weeks 2 and 6 ($p<0.05$), reaching a peak of around 120 mg/L at week 6. The results obtained, in particular the kinetics of biochemical markers, confirmed the relevance of this model for assessing the mechanisms of bone consolidation. The prospects for future research are vast and promising, paving the way for significant advances in maxillofacial surgery and regenerative medicine.

Keywords: Bone Remodeling, Robustness, Reproducibility, Mouse Model, Mandibular Fracture

RÉSUMÉ

La compréhension fine de la cinétique de la consolidation osseuse mandibulaire nécessite des modèles expérimentaux robustes. Ce travail évalue la robustesse et la reproductibilité d'un modèle de lésions osseuses mandibulaires chez le rat Wistar. L'étude a été menée sur 24 rats Wistar, divisés en trois groupes expérimentaux de 08 rats. Le premier groupe a servi de contrôle. Le deuxième groupe a subi une extraction de l'incisive centrale inférieure avec lésion de l'os alvéolaire sous-jacent. Le troisième groupe a subi des trous de forage au niveau de la symphyse mandibulaire. La sédation des rats a été réalisée par injection intra-péritonéale de kétamine et de diazépam. L'extraction dentaire a été effectuée avec un davier incisif, et les trous de forage avec un micromoteur et un foret en acier, sous irrigation de sérum physiologique. Tous les animaux ont reçu un traitement antibiotique et analgésique post-opératoire. L'activité de la phosphatase alcaline (PAL) a significativement augmenté chez les femelles (289,33 à 437 UI/L, $p<0,01$) et chez les mâles (56,75 à 303,67 UI/L, $p<0,01$), indiquant une réponse ostéogénique plus intense et prolongée avec le trou de forage qu'avec l'extraction dentaire. Chez les femelles, le trou de forage a diminué le calcium à 55,5 mg/L, tandis que chez les mâles, l'extraction

dentaire l'a augmenté à 24,3 mg/L et le forage à 128,25 mg/L, suggérant une réponse de remodelage osseux variable selon le sexe et le type de lésion. Chez les femelles, le phosphore a significativement augmenté dès la première semaine dans le groupe trou de forage, atteignant une valeur moyenne d'environ 100 mg/L, tandis que chez les mâles, l'augmentation progressive du phosphore dans le même groupe s'est produite entre la 2ème et la 6ème semaine, atteignant un pic d'environ 120 mg/L à la 6ème semaine. Les résultats obtenus, notamment la cinétique des marqueurs biochimiques, ont confirmé la pertinence de ce modèle pour évaluer les mécanismes de consolidation osseuse. Les perspectives de recherche future sont vastes et prometteuses, ouvrant la voie à des avancées significatives dans le domaine de la chirurgie maxillo-faciale et de la médecine régénérative.

Mots clés : Remodelage Osseux, Robustesse, Reproductibilité, Modèle Murin, Fracture Mandibulaire.

1. INTRODUCTION

Mandibular bone consolidation is a complex and finely regulated biological process, essential for the restoration of masticatory function and the integrity of the facial skeleton (Einhorn 2005). Mandibular bone lesions, whether of traumatic, surgical, or pathological origin, require a thorough understanding of bone remodeling mechanisms in order to develop effective therapeutic strategies (Festing et al. 1998).

Animal models, in particular murine models, play a crucial role in the study of mandibular bone consolidation, enabling fundamental aspects of osteogenesis, angiogenesis, and inflammation to be explored (Gbetibouo et al. 2018). However, the reproducibility and robustness of these models are essential to guarantee the reliability of results and their clinical translation (Ioannidis 2005). The development of a robust and reproducible murine model of mandibular bone lesions offers several advantages. Firstly, it enables experimental conditions to be standardized, thereby reducing inter-individual variability and improving measurement accuracy (Marsell et al. 2011). Secondly, it facilitates the comparison of results between different studies, thus promoting knowledge accumulation and meta-analysis (Schmidmaier et al. 2009). Thirdly, it offers the possibility of studying the impact of different therapeutic interventions, such as the administration of growth factors or biomaterials, on bone consolidation (Turner et al. 2011)

This study aimed to evaluate the robustness and reproducibility of a Wistar rat model of mandibular bone lesions by examining the kinetics of biochemical markers of bone remodeling.

2. MATERIALS AND METHODS

2.1. Study Population

Type and location of study: Experimental, prospective, controlled study. The surgical treatment was administered to rats in the multidisciplinary laboratory of Galenic Pharmacy and Pharmaceutical Legislation of the FMSB-UYI. Blood analysis was performed at the CHUY Laboratory.

Study duration/period: Study period: February 1 to April 31, 2024. The surgical treatment period lasted 6 weeks, and the blood analysis period extended over 6 weeks post-operatively.

Animals: Male and female Wistar rats (14 male rats and 10 female rats), 08 weeks old, weighing 150 grams minimum.

Rats aged 08 weeks are considered young adults, enabling bone remodeling to be studied in a context of growth and maturation. The minimum weight of 150 grams ensures that the animals are sufficiently developed for surgery and blood sampling.

Number of animals: 24 rats divided into 03 groups:

Group 1 (n = 8): control group that received no surgical treatment. Blood sampling was performed as in all other groups II and III at D1, D7, D14, D28, and D42.

Group 2 (n = 8): rats having undergone dental extraction

Group 3 (n = 8): rats with a bone drill hole

Exclusion criteria: Animals with post-operative complications (infection, pathological fracture).

Note: Weight loss after surgery was not considered an exclusion criterion as it could indicate feeding difficulties related to mandibular robustness.

2.2. Experimental Details

2.2.1. Surgical procedure

Anesthesia: Male Wistar rats were anesthetized by intraperitoneal injection of a mixture of ketamine (80 mg/kg, Imalgene 1000, Merial) and diazepam (10 mg/kg, Valium, Roche). Ketamine is a dissociative anesthetic that induces analgesia and amnesia, while diazepam is a benzodiazepine that induces sedation and muscle relaxation. The combination of these two agents produces a balanced anesthesia with reduced side effects. The depth of anesthesia was assessed by the absence of a paw withdrawal reflex and the maintenance of regular breathing (respiratory rate: 60-80 movements per minute).

Dental extraction technique: An alveolar lesion was induced by extraction of the mandibular incisor using a mandibular bone forceps (Hu-Friedy). This technique was standardized to create a reproducible bone lesion at the level of the mandibular symphysis (lesion size: 2-3 mm in diameter).

Drill hole technique: A 1.6 mm diameter drill hole was drilled into the mandibular symphysis using a surgical steel drill (Straumann) mounted on a Marathon micromotor (Marathon Champion 3) running at a speed of 20,000 rpm. Sterile saline irrigation (NaCl 0.9%, Baxter) was used (flow rate: 5 mL/min) to minimize heat and bone damage during drilling.

Post-operative analgesia: The animals received post-operative analgesia with diclofenac 75mg injection (subcutaneous) immediately after surgery and every 12 hours for the first 72 hours.

Antibiotic prophylaxis: A single dose of amoxicillin (50 mg/kg, Clamoxyl, GSK) was administered intramuscularly immediately after surgery as antibiotic prophylaxis.

2.2.2. Blood sampling

Time of sampling: Blood samples were collected by retro-orbital puncture on postoperative days 1, 7, 14, 28, and 42.

Collection route: Blood samples were collected by retro-orbital puncture, using heparinized capillaries (Microvette CB 300 LH, Sarstedt). A new capillary was used for each collection to avoid sample contamination.

Sample preservation: Serum was separated by centrifugation (3000 rpm, Jouan CR4i centrifuge, Thermo Scientific) for 10 minutes at 4°C and stored at -80°C (Revco ULT freezer, Thermo Scientific) until analysis.

2.2.3. Biochemical analysis

Markers measured: Serum levels of alkaline phosphatase (ALP), calcium (Ca), and phosphorus (P) were measured using colorimetric and enzymatic methods.

Analysis techniques

Calcium: Serum calcium was determined by a colorimetric method using the o-cresolphthalein-calcium complex, where the intensity of the coloration formed is proportional to the calcium concentration (Biolabo Kit Ref 27202). This method is based on the formation of a stable complex between calcium ions and o-cresolphthalein in an alkaline medium.

Phosphorus: The concentration of inorganic phosphorus was determined by a colorimetric reaction involving the formation of a phosphomolybdate complex, whose absorbance was measured at 340 nm (Kit Biolabo Ref 27222). This method relies on the reaction of phosphate ions with ammonium molybdate in acidic conditions.

Alkaline phosphatase (ALP): Alkaline phosphatase activity was measured by a kinetic enzymatic method using p-nitrophenylphosphate as substrate, where the rate of p-nitrophenol formation is proportional to enzyme activity (Kit Biolabo Ref 27242). This method measures the enzyme's ability to hydrolyze the substrate at pH 10.4.

Instruments used: Absorbances were measured using a UV-Visible spectrophotometer (Spectrophotometer UV-1800, Shimadzu). Readings were taken at 405 nm for ALP, 570 nm for Calcium, and 340 nm for Phosphorus.

2.3. Study variables

Independent variables: Experimental groups, post-operative time (D1, D7, D14, D28, D42).

Dependent variables: Serum levels of Alkaline Phosphatase (ALP) (U/L), Calcium (Ca) (mmol/L), Phosphorus (P) (mmol/L).

2.4. Statistical analysis

Statistical software: Graph Pad Prism version 8.0.1 (Graph Pad software, San Diego, California, USA) was used for all statistical analyses.

Statistical tests: Repeated-measures ANOVA (mixed model) was performed to assess differences between groups over time, followed by Tukey test for post-hoc multiple comparisons.

Significance level: $p < 0.05$ was considered statistically significant.

2.5. Ethical considerations

Ethics committee approval: Our study obtained prior ethics approval (N° 854 CIER/UY1/FMSB/VDRC/DAASR/CSD).

Compliance with principles: The Marshall Hall Principles, the Principles of Laboratory Animal Care (Guide for the Care and Use of Laboratory Animals, 8th edition) and the 3R Rule (Replacement, Reduction, Refinement) were scrupulously followed.

Animal welfare: Daily monitoring of animals was performed, with administration of analgesics and provision for humane euthanasia in the event of complications.

3. RESULTS

3.1 Effect of induced bone lesions on alkaline phosphatase activity

In females, a significant increase in ALP activity was observed at week 4 (289.33 IU/L, $p < 0.01$), week 5 (387 IU/L, $p < 0.001$) and week 6 (437 IU/L, $p < 0.001$) in both groups II and III. In males, ALP activity in the drill hole group (group III) was significantly higher than in the tooth extraction group (group II) at week 1 (135.67 IU/L vs. 56.75 IU/L, $p < 0.01$), week 2 (177 IU/L vs. 84 IU/L, $p < 0.01$), week 3 (211.25 IU/L vs. 135.5 IU/L, $p < 0.05$) and week 5 (303.67 IU/L vs. 177.75 IU/L, $p < 0.01$).

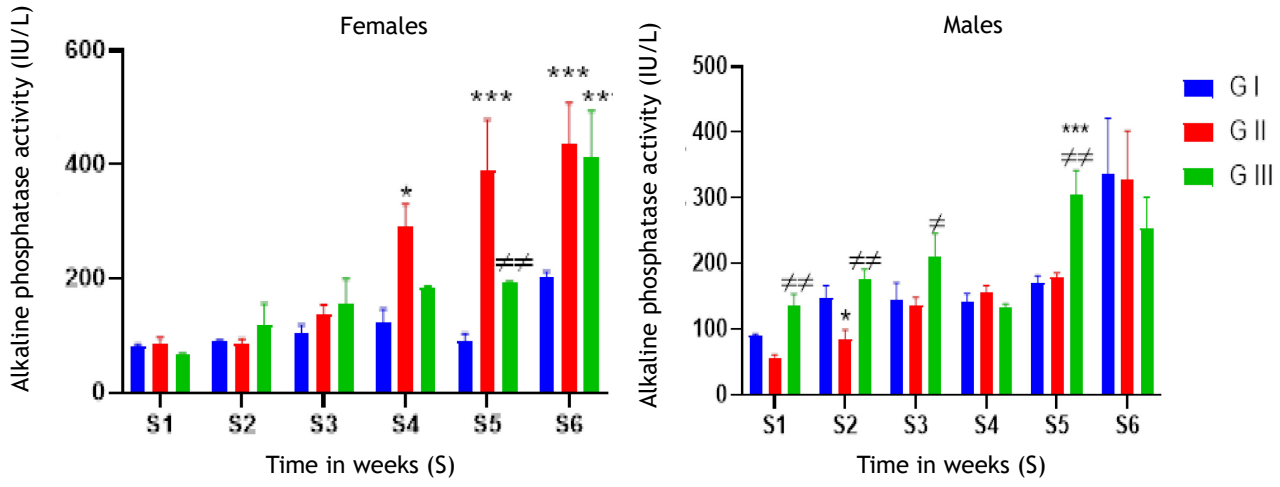

Figure 1: Effects of tooth extraction and drilling on alkaline phosphatase activity in female (n=5) and male (n=8) rats. Legend: S1= week 1, S2= week 2, S3= Week 3, S4= week 4, S5= Week 5, S6= Week 6. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$: ≠≠ $p < 0.01$ G I= control, G II= tooth extraction, G III= drill hole

3.2. Effect of induced bone lesions on calcium activity

In females, the drill hole resulted in a significant decrease in calcium levels at week 5 (55.5 mg/L, $p < 0.05$) compared with the control group (107.67 mg/L). In males, tooth extraction induced a significant increase in calcium levels (24.3 mg/L, $p < 0.05$) at week 2 compared with the control group (18.33 mg/L). The drill hole also resulted in significant increases in calcium levels in males at weeks 2 through 6, with the highest level at week 6 (128.25 mg/L, $p < 0.05$).

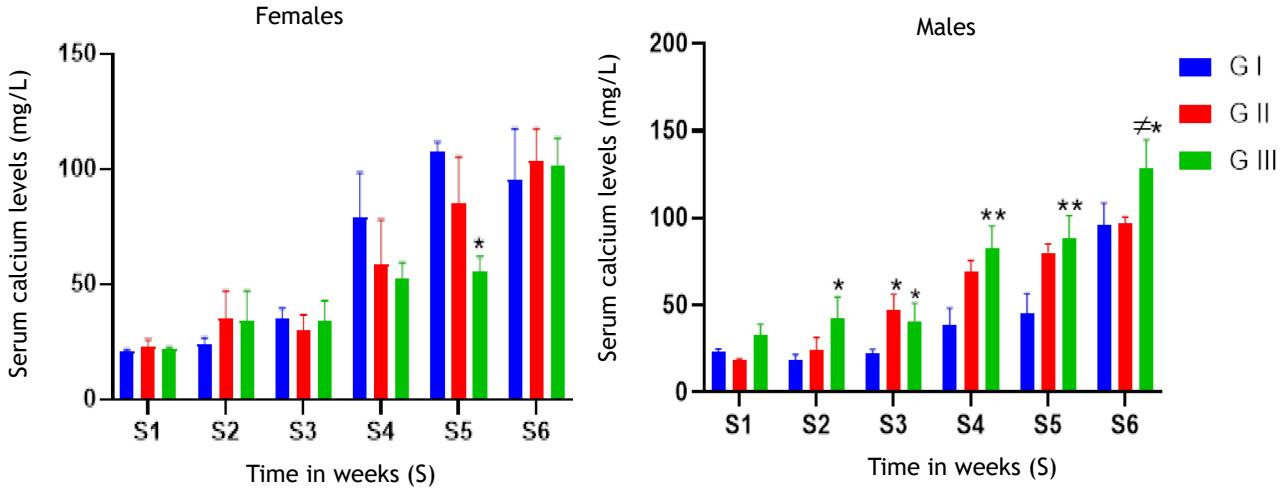

Figure 2: Effects of tooth extraction and drilling on calcium activity in female (n=5) and male (n=8) rats. Legend: S1= week 1, S2= week 2, S3= Week 3, S4= week 4, S5= Week 5, S6= Week 6. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$: ≠≠ $p < 0.01$ G I= control, G II= tooth extraction, G III= drill hole

3.3. Effect of induced bone lesions on phosphorus activity

In females, phosphorus levels increased significantly from week 1 in the drill hole group (GIII), reaching a mean value of around 100 mg/L ($p < 0.05$). In males, the increase in phosphorus in the GIII group occurred progressively between weeks 2 and 6, reaching a peak of around 120 mg/L at week 6 ($p < 0.05$).

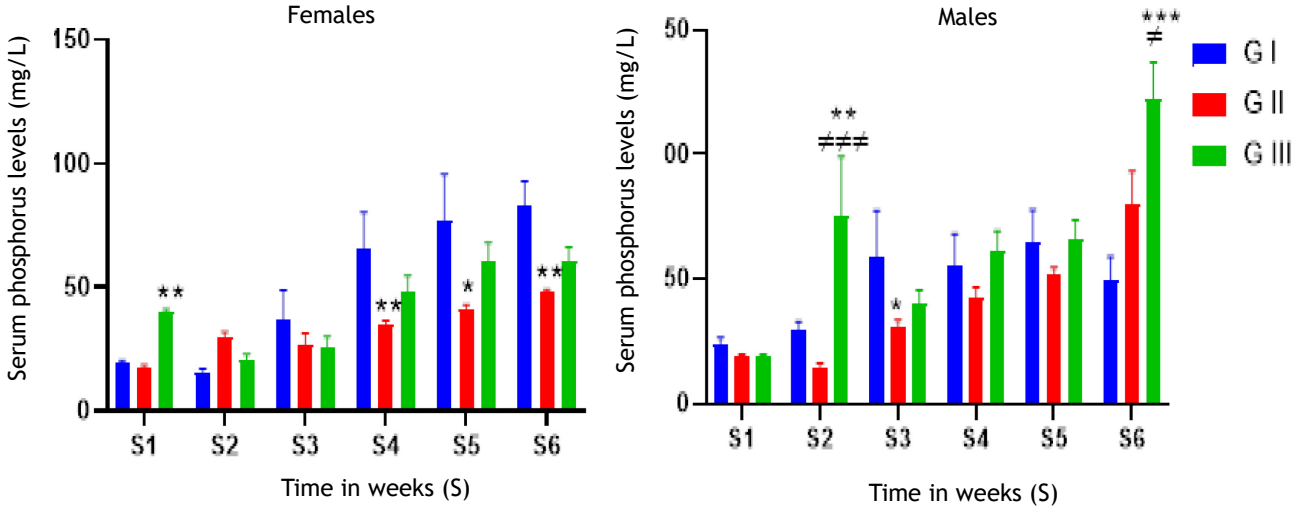

Figure 3: Effects of tooth extraction and drilling on phosphorus activity in female (n=5) and male (n=8) rats. Legend: S1= week 1, S2= week 2, S3= Week 3, S4= week 4, S5= Week 5, S6= Week 6. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$: ≠≠ $p < 0.01$ G I= control, G II= tooth extraction, G III= drill hole

4. DISCUSSION

4.1 Effect of induced bone damage on alkaline phosphatase (ALP) activity

The significant increase in serum ALP activity observed in both treatment groups reflects the activation of osteoblastic activity during bone remodeling. This elevation in ALP levels indicates enhanced bone formation processes, with drilling inducing a more pronounced and sustained osteogenic response compared to tooth extraction. The progressive increase from weeks 4 to 6 suggests ongoing bone matrix synthesis and mineralization.

Our results are similar to those of Nilajagi et al. (2021), who observed a significant increase in ALP from postoperative days 3 to 30. According to these authors, this could be due to osteoblastic proliferation at the fracture site and the contribution of the periosteum of the destroyed bone, a rich source of alkaline phosphatase.

These findings confirm the sensitivity of our murine model to detect changes in ALP activity, a key marker of bone formation, which is essential for assessing the robustness and reproducibility of the model.

4.2 Effect of induced bone damage on calcium activity

The differential calcium response observed between sexes and treatment groups reflects the complex mineral homeostasis during bone remodeling. In males, the sustained elevation of serum calcium levels, particularly in the drill hole group, suggests increased mobilization of calcium from bone stores to support the healing process. The early peak at week 2 in the tooth extraction group likely represents the initial inflammatory response and calcium release from damaged alveolar bone. In contrast, the decrease observed in females may indicate enhanced calcium uptake at the fracture site during the active mineralization phase.

The increase in serum calcium was observed in Group II males at week 3. In group III, this increase was observed between weeks 2 and 6. This could be explained by the fact that calcium was secreted into the bloodstream and delivered to the fracture site to consolidate and stiffen the damaged bone.

Indeed, the mineral matrix is responsible for bone rigidity, with minerals binding to the protein structure of the osteoid for mineralization (Boskey 1992). Mineralization (the third phase of fracture healing) of the soft callus occurs through the deposition of minerals such as calcium and phosphorus on the newly formed matrix (Einhorn 2005). Calcium and phosphorus are necessary for bone formation and are stored in the bone in the form of hydroxyapatite. They give bone its rigidity.

These variations in serum calcium demonstrate the ability of our model to reflect the mineral fluctuations associated with bone remodeling, reinforcing its robustness.

4.3 Effect of induced bone damage on phosphorus activity

The gender-specific kinetics of phosphorus metabolism observed in our study reveals important insights into bone remodeling patterns. The earlier response in females (week 1) compared to the more gradual increase in males (weeks 2-6) suggests sex-related differences in metabolic responses to bone injury. The sustained elevation of phosphorus levels indicates active mineralization processes, with phosphorus being essential for hydroxyapatite crystal formation during bone healing.

Results obtained in the present study on serum phosphorus levels showed a significant increase in this parameter in Group III females at week 1. In males of the same group, the increase occurred between weeks 2 and 6. This observation was also made in Group II of our experiment. In fact, high levels of calcium and phosphorus in the blood and extracellular fluids trigger the deposition of calcium phosphate crystals in the osteoid, making the bone harder (Marie 2003). Phosphorus is therefore an essential bone component required for proper skeletal mineralization. Most of the body's phosphorus is stored in bone (Bronner 1992)

The phosphorus kinetics observed in our study highlight the model's sensitivity to variations in this mineral crucial to bone formation, thus validating its reproducibility and its potential for advancing our understanding of bone remodeling mechanisms.

5. CONCLUSION

This study demonstrates that the murine model of mandibular bone lesions constitutes a pivotal investigative paradigm for elucidating the intricacies of bone remodeling and evaluating innovative therapeutic modalities. The

significant elevation of alkaline phosphatase activity, coupled with the differential calcium and phosphorus responses between sexes and treatment types, confirms the model's robustness and reproducibility. The mouse model of mandibular bone lesions is a valuable tool for studying bone remodeling and evaluating new therapeutic strategies. The kinetics of biochemical markers observed in this study provide crucial insights into the temporal patterns of bone healing, with drilling procedures inducing more intense and prolonged osteogenic responses compared to tooth extraction. Future research prospects are vast and promising, paving the way for significant advances in maxillofacial surgery and regenerative medicine.

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CONFLICTS OF INTEREST. The authors declare that they have no known competing financial interests or personal relationships that might appear to influence the work reported in this article. The authors declare that they have no financial interests/personal relationships that could be considered as potential competing interests.

AUTHOR CONTRIBUTIONS. NKOLO TOLO FD. And NKECK J.R designed the study. ATANWO DONGMO N.L. and TANETCHOP N. collected the data. EKO M. and OBONO EKAMENA MJ. carried out the statistical analysis and drafted the manuscript. NJIKI BIKOI J. critically read the manuscript. All authors have given their consent for publication.

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