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Refined Surgical Protocol for the Insertion of Bioactive-coated Titanium Microscrews in the Rat Tibia

ALEXIS VERA-BECERRA[1,2], IVÁN VALDIVIA-GANDUR[3], PABLO ACUÑA-MARDONES[2,4], FRANCISCA ACEVEDO[5,6,7], CARLOS VEUTHEY[8] and VÍCTOR BELTRÁN[2,6]

[1]Master's Program in Dentistry, Faculty of Dentistry, Universidad de La Frontera, Temuco, Chile;
[2]Clinical Research and Dental Innovation Center (CIDIC), Faculty of Dentistry, Universidad de La Frontera, Temuco, Chile;
[3]Biomedical Department, Universidad de Antofagasta, Antofagasta, Chile;
[4]PhD Program in Morphological Sciences, Universidad de La Frontera, Temuco, Chile;
[5]Department of Basic Sciences, Faculty of Medicine, Universidad de La Frontera, Temuco, Chile;
[6]Center of Excellence in Translational Medicine (CEMT) and Scientific and Technological Nucleus in Bioresources (BIOREN), Universidad de La Frontera, Temuco, Chile;
[7]Millennium Nucleus for Bioproducts, Genomics and Environmental Microbiology (BioGEM), Valparaíso, Chile;
[8]Center of Excellence in Morphological and Surgical Studies (CEMyQ), School of Medicine, Universidad de La Frontera, Temuco, Chile

Abstract

Background/Aim: Sensitive bioactive coatings, such as polycaprolactone with cholecalciferol, present handling challenges in animal models due to their susceptibility to mechanical damage. This study aimed to develop and validate a refined surgical protocol for inserting such polycaprolactone-cholecalciferol-coated titanium microscrews into the proximal tibia of Sprague-Dawley rats, ensuring primary stability and preserving the integrity of the nanofibrous coating.

Materials and Methods: Fourteen male Sprague-Dawley rats (300-350 g) were used: one pilot and 13 animals in the main trial. A 3D anatomical tibia model was created for *in-vitro* validation of the surgical technique. The refined protocol incorporated enlarged cortical drilling (1.8 mm) before inserting microscrews (1.5×7 mm) to minimize friction on the outer cortical bone. Multimodal anesthesia, postoperative analgesia, and systematic clinical monitoring were implemented. Coating integrity, primary stability, wound healing, and animal welfare were evaluated through clinical observation, micro-computed tomography, and scanning electron microscopy (SEM).

Results: The refined protocol achieved 100% procedural success. All implants reached immediate primary stability with uneventful first-intention healing and no critical adverse events. SEM confirmed preservation of morphology of the nanofibrous coating exclusively in the refined protocol group. Micro-computed tomography demonstrated consistent bone-to-implant contact in both cortical and trabecular compartments. Clinical recovery was rapid and spontaneous, with all animals maintaining optimal welfare indicators throughout follow-up.

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Víctor Beltrán, Avenida Francisco Salazar 01145, Temuco 4780000, Chile. Tel: +56 452734153, e-mail: victor.beltran@ufrontera.cl and Iván Valdivia-Gandur, Avenida Angamos 601, Antofagasta 1270300, Chile. e-mail: ivan.valdivia@uantof.cl

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Conclusion: The refined protocol safeguards the structural integrity of functionalized surfaces, ensures primary stability, and promotes early osseointegration, while complying with the principles of replacement, reduction, and refinement (3Rs). This model provides a valid experimental platform for investigations into osseointegration dynamics and the controlled local release of therapeutic agents.

Keywords: Animal models, biocompatible coatings, titanium, tibia, osseointegration, preclinical studies.

Introduction

The development of functionalized implants with bioactive coatings has opened new possibilities in regenerative medicine and implantology. Among these, electrospun polymeric coatings enabling the controlled release of therapeutic agents, such as vitamin D, have shown promising effects under both *in-vitro* and *in-vivo* conditions (1, 2). However, their clinical translation requires rigorous preclinical validation in appropriate animal models that ensure the integrity of the coating during insertion and allow assessment of its performance in the actual biological environment (3).

Preclinical evaluation of implantable coatings requires the selection of suitable animal models. Although rabbits have often been used because of their availability and intermediate size (4), their use has decreased in favor of more refined models or *in-vitro* alternatives (5). Nevertheless, their historical application in osseointegration studies, including the validation of new bioactive surfaces, has been key in the development of modern implantology (6). Relevant criteria for model selection include reproducibility, tolerance to captivity, cost-effectiveness, and anatomical accessibility (6).

Various anatomical sites have been reported in the literature for evaluation of implantable materials, each with advantages and limitations. The femur, for example, offers good surgical accessibility and bone volume but its soft marrow limits primary stability for short implants (7). The mandible more closely resembles the oral environment but involves more complex surgeries and a higher risk of affecting vital structures (8). The skull allows wide and flat access for histological analysis but its thinness limits the insertion of functionally sized implants (9, 10). In addition to these limitations, a key technical challenge is that the friction generated during insertion can compromise nanofibrous coatings, which are highly sensitive to detachment or mechanical damage (2, 9).

In this context, the proximal tibia of the Sprague-Dawley rat has been validated as a suitable model for studying bone integration of micro-implants, as it allows transverse insertion with controlled surgical access, low risk of complications, and the possibility of radiographic, histological, and electron microscopy analyses (11, 12). It is also compatible with modern surface-modification techniques incorporating sustained-release systems for osteoactive elements such as strontium or cholecalciferol, which have been shown to enhance osseointegration even in compromised bone conditions (13-15). However, conventional direct insertion protocols do not consider protection of the surface coating, compromising the reproducibility of results when bioactive platforms are involved (3, 15).

Furthermore, the ethical design of animal models is of growing importance. International animal welfare guidelines promote the principles of replacement, reduction, and refinement (the 3Rs), prioritizing less invasive surgical models, with lower variability and higher data quality (16-18). In this context, the exclusive use of male rats responds to the need to control the hormonal influence of the estrous cycle in females, which has been documented as a relevant factor in bone regeneration and bone biomechanics (19, 20).

This study proposes and validates a refined surgical model for the insertion of titanium microscrews coated with polycaprolactone and cholecalciferol, evaluating

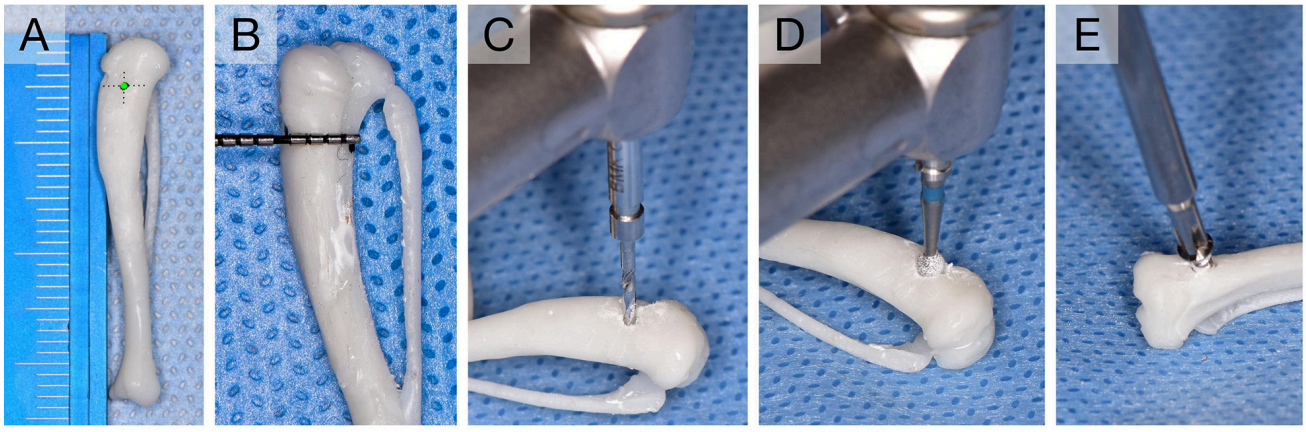

Figure 1. *In-vitro simulation of the refined surgical protocol using a 3D-printed model of a rat tibia. (A) Anatomical overview of the 3D-printed tibia with metric scale for dimensional validation. (B) Positioning and identification of the entry point for microscrew insertion. (C) Pilot drilling of 1.5 mm in the metaphyseal region. (D) Subperiosteal cortical enlargement with a 1.8 mm round bur. (E) Manual insertion of the microscrew using a surgical driver.*

their structural integrity and potential applicability in preclinical studies. Through a modification in the design of the recipient bed, the aim is to preserve the bioactive coating during insertion, ensure primary stability in the opposite cortical bone, and establish a reproducible, ethical model compatible with advanced bone analysis techniques.

Materials and Methods

Presurgical modeling and in-vitro simulation. As a complement to the experimental study and with the aim of reinforcing the reproducibility of the refined protocol, an *in-vitro* simulation was developed using a three-dimensional (3D) anatomical model of the rat tibia. This tool was implemented in the final phase of the study to visually document the technical sequence and retrospectively validate the suitability of the surgical instruments for the bone geometry of the animal model.

The 3D model was generated from an STL file corresponding to the right tibia of an adult Wistar rat, obtained from an open-access digital repository (MorphoSource; https://www.morphosource.org). The dimensions of the model were verified to be compatible with the Sprague-Dawley strain used in the study. Printing was performed using a photopolymer resin and a 2K resolution LCD technology (LD-002R; Creality 3D, Shenzhen, PR China), enabling accurate reproduction of cortical contours and metaphyseal proportions.

A complete replica of the refined surgical technique was performed on this model, including photography with a contrasting background for anatomical relief assessment, documentation of the surgical site with a calibrated metric scale, sequential drilling tests (pilot drill of 1.5 mm followed by a round drill of 1.8 mm), and manual insertion of the microscrew using a surgical driver (Figure 1).

Experimental animals. Fourteen male Sprague-Dawley rats (weight 300-350 g, age 9-12 weeks) were used. The animals were obtained from the Experimental Surgery and Animal Facility Center (Centro de Cirugía Experimental y Bioterio), Universidad de La Frontera, Temuco, La Araucanía Region, Chile. Animals were housed under standard animal facility conditions with controlled temperature (20-24°C), relative humidity (35-70%), and a 12-h light/dark cycle, with *ad libitum* access to food and water. The exclusive use of males was justified by the need to avoid variability induced by the estrous cycle, widely documented as a determining factor in bone remodeling, mineral density, and the response to osteoinductive biomaterials (1, 2).

All procedures were performed in accordance with ARRIVE guidelines (21) and in compliance with the Declaration of Helsinki and current international standards on animal experimentation. The protocol was approved by the Research Ethics Committee of the Universidad de La Frontera, Chile (Act No. 139_2024). Measures were taken to reduce animal suffering and optimize experimental design, in line with the principles of the 3Rs.

Implant preparation. Grade V titanium (Ti-6Al-4V) microscrews (Biomaterials Korea, Inc., Seoul, Republic of Korea) measuring 1.5×7 mm were used, selected for their proven biocompatibility, superior mechanical strength, and adequate osteoblastic response in animal models. The microscrews are produced in Ti-6Al-4V ELI grade, in accordance with ASTM F136-13 standards, ensuring consistency in composition and mechanical performance. Each implant was functionalized with a bioactive coating composed of a polymeric matrix of polycaprolactone at 14% w/w, incorporating cholecalciferol (vitamin D_3) as an osteoactive agent, obtained by electrospinning. This method enabled the formation of a continuous nanofibrous layer, uniformly adhered to the screw surface, with controlled thicknesses of 25 and 50 μm, providing sustained-release properties and structural mimicry of the bone extracellular matrix (1).

To preserve sterility without compromising the physicochemical integrity of the bioactive coating, a controlled ultraviolet (UV) irradiation procedure was applied. The coated microscrews were exposed to UV light at 254 nm for 30 min on each side inside a sterile laminar flow cabinet immediately prior to surgery. This ensured asepsis while maintaining the morphological integrity of the polymeric nanofibers.

UV irradiation was selected instead of autoclaving or ethylene oxide sterilization, as those methods are known to alter the morphology and biofunctionality of polycaprolactone-based electrospun fibers. The validity of this method had been previously confirmed by scanning electron microscopy (SEM) morphological evaluation and cell-viability assays, which demonstrated that the coating preserved its fibrous structure and bioactive capacity following UV exposure (4). This procedure ensured the structural preservation and biofunctionality of the coating during pre-surgical storage.

Presurgical validation test of the protocol. Prior to the main study, an exploratory test was carried out on a male Sprague-Dawley rat (350 g) to experimentally validate the impact of the surgical technique on the integrity of the bioactive coating. Two polycaprolactone-coated microscrews were implanted, one in each proximal tibia, under standardized conditions of anesthesia, asepsis, and surgical handling. The left tibia (Protocol 1) underwent conventional insertion with a 1.5 mm pilot drill, without cortical enlargement. The right tibia (Refined protocol) was prepared using a 1.5 mm pilot drill plus subperiosteal cortical drilling with a 1.8 mm round bur.

After 24 h, the animal was euthanized, and both tibiae were retrieved and prepared for surface analysis by SEM. Samples were dehydrated and mounted for observation. In addition, the insertion and macroscopic condition of the implants at the time of removal were photographically documented.

This trial allowed comparison of the structural response of the coating under two different operative conditions and supported the implementation of the refined protocol in the main experimental design.

Main study groups. Rats were divided into two experimental groups according to the thickness of the electrospun polycaprolactone-cholecalciferol layer applied to the titanium microscrews: 25 μm (n=6) and 50 μm (n=7).

Refined surgical protocol. A refined surgical protocol was established for systematic application in the main study. Under multimodal anesthesia (100 mg/kg ketamine and 13 mg/kg xylazine administered intraperitoneally, plus inhaled sevoflurane at 250 mg), a 15-mm longitudinal incision was made over the anteromedial surface of the

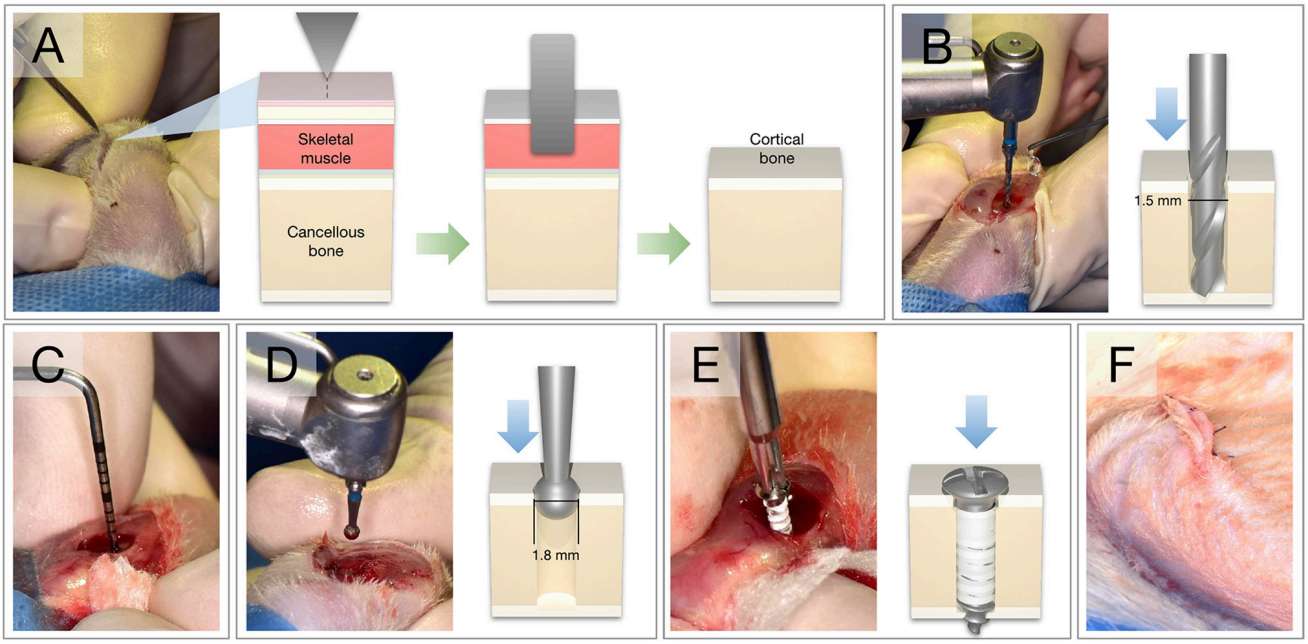

Figure 2. *Operative sequence of the refined surgical protocol. (A) Longitudinal incision of 15 mm over the proximal tibia, followed by blunt dissection and exposure of the cortical bone. (B) Pilot drilling of 1.5 mm using BMK® kit instruments. (C) Measurement of the depth of the surgical bed. (D) Enlarged subperiosteal cortical drilling with a 1.8 mm diamond round bur. (E) Manual insertion of the bioactive-coated microscrew. (F) Layered closure with absorbable suture and application of topical antibiotic.*

proximal tibia. Blunt dissection of the planes was performed with minimal tissue traction, allowing full exposure of the cortical bone.

The surgical approach incorporated a deliberate technical modification from the traditional model: a wider initial bone bed was created in the outer cortical bone using sequential drilling with a 1.5 mm pilot drill (BMK® surgical kit, Neobiotech Co., Ltd., Seoul, Republic of Korea) and a 1.8 mm round diamond bur connected to a surgical motor with controlled torque (20-30 N cm). This maneuver aimed to minimize initial friction of the coating against bone, thereby preventing detachment, folding, or compression during insertion (Figure 2).

The implant was manually introduced using the system's surgical driver, allowing control of axial pressure without applying excessive torque on the polymer structure. Final fixation was achieved by contact with the inner cortical bone, ensuring primary stability without compromising coating integrity.

Closure was performed in layers with absorbable 5-0 suture, and topical 2% chlortetracycline (Pederol®) was applied prophylactically.

Postoperative management. Enrofloxacin (5 mg/kg subcutaneously) was administered 1 h before the procedure and ketoprofen (5 mg/kg subcutaneously) every 24 h for 3 days. Rats were monitored daily during the first 2 weeks, and then every 72 h. Clinical parameters of wound healing, inflammatory signs, and adverse events were recorded.

At the endpoint, animals were euthanized *via* an overdose of ketamine hydrochloride (100 mg/kg; Ketamil®, Troy Laboratories Pty Ltd., Glendenning, NSW, Australia) combined with xylazine hydrochloride (10 mg/kg; 2% Xilazina, Centrovet, Santiago, Chile) administered intraperitoneally, followed by bilateral pneumothorax as a secondary method to ensure a humane death. Tibiae were carefully dissected, preserving the peri-implant region for subsequent microtomographic and surface analyses.

Micro-computed tomography (micro-CT). High-resolution scans were performed using a SkyScan 1273 system (Bruker, Kontich, Belgium) at 9 μm voxel size, 60 kV, 200 μA, with a 0.5 mm aluminum filter. The images were reconstructed and analyzed using Dragonfly 4.1 (Object Research Systems, Montreal, Canada). The region of interest was defined as a cylindrical volume of interest measuring 1.6 mm in diameter×1.8 mm in height, encompassing both cortical and trabecular compartments. This approach allowed for the qualitative assessment of peri-implant bone morphology and integration in relation to the two coating thicknesses.

Results

Presurgical modeling and in-vitro simulation (retrospective validation). The 3D printing of the right rat tibia enabled the generation of an anatomically representative model, with cortical reliefs and dimensions compatible with the proximal tibial region of the Sprague-Dawley strain. The images obtained show a lateral view of the bone model with a contrasting background, highlighting the main anatomical landmarks of the anteromedial surgical approach (Figure 1).

A calibrated millimetric scale was positioned at the surgical site to validate the dimensional correspondence of the printed model with real measurements, confirming its usefulness as a platform for planning and operative simulation.

The photographic sequence included identification of the screw entry site, initial drilling with a 1.5 mm pilot bur, and cortical widening with a 1.8 mm round bur. Subsequently, the manual insertion of the titanium microscrew was documented using the surgical driver, showing progressive fit without model fracture and with a conservative angulation replicating *in-vivo* operative conditions.

The simulation of the surgical procedure demonstrated the ergonomic compatibility of the instruments with the anteromedial approach and visually validated the sequence of drilling and progressive insertion of the microscrew without model fracture. This retrospective stage allowed the graphical standardization of the protocol, optimization of its technical description, and reinforcement of the applicability of the refined model as a basis for future research.

Presurgical validation trial. The refined protocol was preliminarily validated in an exploratory trial performed on one Sprague-Dawley rat, in which polycaprolactone-coated microscrews were inserted into both tibiae using two technical variants: conventional (without enlarged drilling) and refined (with 1.8 mm subperiosteal cortical drilling). After 24 h, the tibiae were retrieved and analyzed by SEM.

In the tibia treated with the conventional technique, folding and disorganization of the coating architecture were observed, with loss of continuity at the cortical entry zone. In contrast, the implant inserted under the refined protocol showed an intact surface, without tears or signs of coating compression, and with well-preserved fibers along its axis. These observations supported the adoption of the modified protocol for the experimental study (Figure 3).

Refined surgical protocol. The refined surgical protocol was applied to 13 Sprague-Dawley rats, housed in pairs. The mean surgical time was 17±2 min. All microscrews achieved immediate primary stability, with no intraoperative complications or anesthetic mortality.

During postoperative follow-up, all animals (100%) exhibited primary-intention healing, although in three cases, mild signs of scratching or biting at the surgical site were observed without implant exposure. These lesions were managed with reapplication of topical chlortetracycline and daily monitoring, achieving complete resolution without infection or functional impairment (Table I).

Animals showed spontaneous recovery of exploratory behavior within the first 24 h, with normal food and water intake. No systemic signs of discomfort, lethargy, or significant weight loss were recorded (Table II).

Photographic documentation during surgery and subsequent evaluation of the implants confirmed the visible preservation of the nanofibrous coating in all

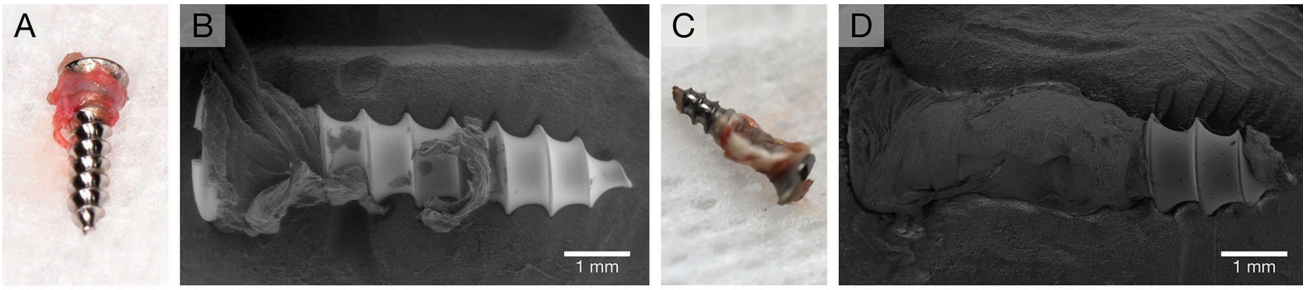

Figure 3. *Comparative analysis of microscrew surface coating after insertion. (A) Macroscopic view of a microscrew inserted with the conventional protocol, revealing partial detachment of the electrospun layer. (B) Scanning electron microscopy image of the same specimen, illustrating the folding and disorganization of nanofibers. (C) Macroscopic view of a microscrew inserted with the refined protocol, displaying a uniform and adherent coating. (D) Scanning electron microscopy image of the same specimen, evidencing preserved nanofibrous architecture without disruption or detachment.*

cases. The implant surface remained continuous, with no alterations in its external morphology. These findings confirm that the refined protocol prevented destructive friction at the entry point and ensured optimal conditions for subsequent functional studies.

Micro-CT analysis revealed peri-implant bone formation in all specimens. Structural integration was evident in 3D reconstructions, showing continuity of bone density between the tibial cortex and the implant surface. In axial and longitudinal sections, a pattern of intimate contact was observed between the newly formed bone and the microscrew, consistent with stable osseointegration.

Bone integration was demonstrated in both the 25 µm coating group (n=6) and the 50 µm coating group (n=7) (Figure 4), although preliminary morphological differences in the adjacent newly formed bone volume were identified. These will be quantitatively assessed in the future.

Table I. *Validation of the refined surgical protocol.*

Indicator	Result
Surgical success	100% (13/13 Animals)
Primary implant stability	100%
Primary intention healing	100% (3 Cases with minor variations)
Critical postoperative adverse events	None
Operative mortality	0%
Visible coating preservation	100%

Table II. *Summary clinical assessment of animal welfare [adapted from Morton and Griffiths (24)].*

Observed parameter	Assessment	Overall result
General appearance	Normal/mild alteration	3 Mild cases
Activity/mobility	Normal	100%
Feeding and hydration	Normal	100%
Surgical wound	Adequate healing	100% (With topical management in 3 cases)
Weight variation	<5%	100%

Discussion

The refinement of preclinical models is essential for improving the translational value of bone regeneration studies while complying with the 3R principles (Table III) (1, 2). In this study, a rat tibia model for the insertion of bioactive-coated titanium microscrews was optimized to preserve the integrity of a nanofibrous polycaprolactone-cholecalciferol coating during surgical placement. The incorporation of a cortical widening step minimized mechanical friction, preventing coating delamination and ensuring primary stability, an aspect often neglected in conventional protocols (3, 4). In fact, traditional approaches relying solely on self-tapping insertion often generate uncontrolled shear forces that compromise delicate surface features, potentially masking the true biological response of bioactive coatings. By reducing this artifact, our refinement ensures a more faithful evaluation of the material-tissue interface.

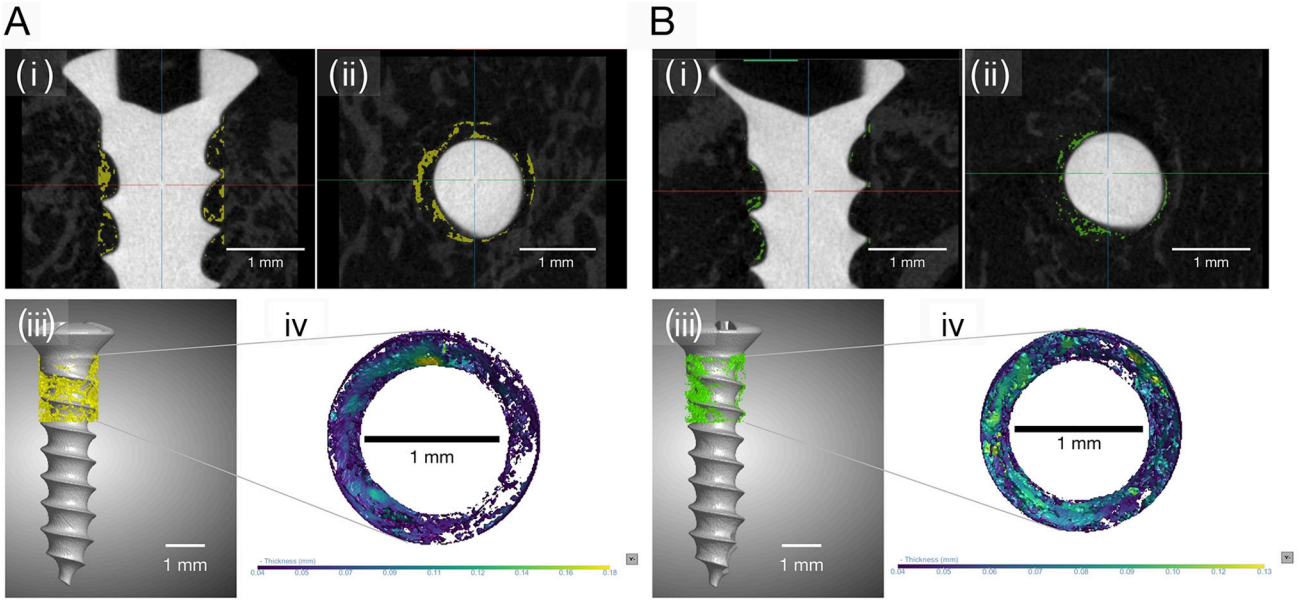

Figure 4. *Evaluation of titanium microscrews after implantation into rat tibia. Microscrews with 25 μm (yellow) (A) or 50 μm (green) (B) electrospun polycaprolactone–cholecalciferol coatings were implanted into rat tibia. Longitudinal (i) and transverse (ii) micro-computed tomography views, showing segmented peri-microscrew tissue around screws. 3D reconstruction of peri-microscrew tissue (iii) and corresponding tissue thickness map (iv) viewed from above. The color scale indicates local tissue thickness (mm). Osseointegration was evaluated within a cylindrical volume of interest of 1.6 mm in diameter and 1.8 mm in height.*

Table III. *Application of the 3Rs principle in the experimental model.*

Principle	Specific application
Replacement	Use of 3D-printed tibia for operative simulation and *in vitro* technical validation
Reduction	Adjusted experimental design; prior validation allowed optimization of final number.
Refinement	Low-friction surgical technique; daily clinical monitoring; timely topical intervention.

Beyond the intraoperative refinement, a retrospective pre-surgical model was used after the *in-vivo* procedures. This step served as an additional validation tool, educational resource for training and future applications, and graphic support for the protocol. By visually demonstrating the technical sequence and outcomes, it reinforced methodological clarity and promoted reproducibility, contributing to the standardization of subsequent research in similar animal models. This approach not only minimizes the need for repeated animal experimentation but also provides a didactic tool to train researchers in a standardized, low-variability technique.

Our results reinforce previous evidence showing that micro- and nanostructured titanium surfaces enhance bone-implant integration by promoting osteoconduction and favorable cell-material interactions (5-7). Comparable biological principles were reported by Senoo *et al.*, who demonstrated that microporous titanium meshes with 50-μm pores facilitated angiogenesis and osteoconductivity in a rat calvarial guided bone regeneration model, outperforming macroporous designs in bone volume and vascular penetration (22). These findings support the notion that preserving delicate surface architecture can directly influence vascularization and early osseointegration.

Micro-CT analysis qualitatively demonstrated peri-implant bone formation and continuity between the cortical bone and implant surface, supporting the descriptive validation of the refined model, consistent with other studies using rat tibia and femur models for early osseointegration assessment (9-11). The choice of

the proximal tibia provided a favorable cortical and trabecular environment for standardized implant positioning, enabling bilateral placement and reducing animal numbers in line with ethical optimization (2, 12).

Physical stimulation modalities, such as photo-biomodulation, have also been proposed to improve bone repair, especially under compromised conditions. Furukawa *et al.* reported that diode laser irradiation significantly increased new bone formation in tibial defects of both estrogen-deficient and normal rats (23). Although our study did not apply adjunctive laser therapy, the principle of reducing surgical trauma while enhancing osteogenic potential is consistent with our low-impact drilling approach.

Limitations of this study include the short observation period and absence of functional loading, which may affect long-term remodeling outcomes (14). Future investigations should incorporate histomorphometric and molecular analyses, extended follow-up, and potential synergy with growth factors, laser therapy, or scaffold systems to further enhance clinical translation. Importantly, maintaining the integrity of the coating during implantation opens the possibility of correlating ultrastructural preservation with histological and molecular endpoints, which has often been hampered in prior models due to procedural damage.

Application of this refined protocol represents a substantial improvement in terms of technical reproducibility, reduction of artifacts, and ethical alignment with *in-vivo* experimentation principles, constituting a solid platform for functional evaluation of implantable surfaces with sensitive bioactive coatings. Beyond serving as a reliable preclinical tool, this model may accelerate the translation of novel bioactive materials to clinical applications, particularly in challenging scenarios such as compromised bone quality, immediate implant placement, or regenerative procedures where surface preservation is critical. Altogether, this dual strategy of operative refinement and retrospective modeling provides both a robust research platform and an educational framework, strengthening the translational bridge between preclinical experimentation and clinical innovation.

The refined surgical protocol presented in this study proved to be an effective, reproducible, and ethically robust approach for the insertion of titanium micro-implants coated with sensitive bioactive materials in the tibia of Sprague-Dawley rats. The modification of the bone bed through enlarged cortical drilling preserved the integrity of the nanofibrous coating during insertion, overcoming one of the main limitations of conventional models.

The findings, supported by morphological observations (SEM) and micro-CT, validate the primary stability of the implant, early osseointegration, and the maintenance of animal welfare, in accordance with the 3R principles in biomedical research. Furthermore, the pre-surgical *in-vitro* simulation using a 3D-printed model contributed significantly to the standardization of the technique and the reduction of experimental variability.

Conclusion

This refined model provides a valid preclinical platform for future research in bone regeneration, controlled delivery of therapeutic agents, and functional evaluation of novel implantable surfaces, being particularly relevant in contexts where the structural sensitivity of the coating determines the reliability of the *in-vivo* trial. Its implementation supports the transition toward more sustainable, precise, and ethically responsible protocols in the study of advanced biomaterials.

Conflicts of Interest

The Authors declare no conflicts of interest in relation to this study.

Authors' Contributions

Alexis Vera-Becerra: Conceptualization, methodology, writing – original draft, formal analysis, data curation, visualization (surgical photography), supervision. Iván Valdivia-Gandur: Surgical execution, methodology, investigation, validation, writing – review and editing.

Carlos Veuthey: Veterinary supervision, animal welfare oversight, surgical assistance, and methodological validation of all *in-vivo* procedures, in coordination with Dr. Valdivia-Gandur. Pablo Acuña-Mardones: Visualization (schematic diagrams), data curation, writing – review and editing. Francisca Acevedo: Resources, project administration, writing – review and editing. Víctor Beltrán: Conceptualization, supervision, funding acquisition, writing – review and editing. All Authors reviewed and approved the final version of the manuscript.

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Artificial Intelligence (AI) Disclosure

During the preparation of this manuscript, a large language model (ChatGPT, OpenAI) was used solely for language editing and stylistic improvements in select paragraphs. No sections involving the generation, analysis, or interpretation of research data were produced by generative AI. All scientific content was created and verified by the authors. Furthermore, no figures or visual data were generated or modified using generative AI or machine learning–based image enhancement tools.

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