A STUDY ON GENTAMICIN IMPREGNATED BIOMATERIALS FOR TREATING INDUCED OSTEOMYELITIS IN THE NEW ZEALAND WHITE RABBIT ANIMAL MODEL: AN OVERVIEW OF MICROSCOPIC ANALYSIS

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Abstract. Osteomyelitis (OM) treatment remains a significant challenge in orthopaedics surgery. This infection is challenging to treat and requires prolonged antibiotic administration. The New Zealand White Rabbit (NZWR) is a suitable experimental model for studying local delivery antibiotics treatment for osteomyelitis because it closely mimics the disease process in humans. This study aimed to induce osteomyelitis in rabbit femurs and analyse the treatment with gentamicin beads impregnated with biomaterials. The study was evaluated in thirty-six of NZWRs. They were divided into Hydroxyapatite (HA) and Calcium Sulphate ($CaSO_4$) with four subgroups: 3, 6, 12, and 26 weeks. Each NZWR underwent two surgeries; involved the first surgery was to induce osteomyelitis by inoculating *Staphylococcus aureus* in the distal femur, followed by the second surgery was for debridement and biomaterial-impregnated antibiotics implantation. Histological interpretations indicated that all rabbits developed osteomyelitis 3 weeks after the bacteria were inoculated. At 6 to 26 weeks, complete healing of the infected area was noted, with the appearance of new bone formation. Both findings indicated a complete bone healing after a 26 weeks interval. The results of histology interpretation in each group were comparable. Therefore, the findings of this study indicated that gentamicin impregnated with HA could be used in treating OM.

Keywords: Osteomyelitis, gentamicin-impregnated biomaterials, histological interpretation, hydroxyapatite, calcium sulphate

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Introduction

Treatment of OM is still a major problem in orthopaedic surgery. OM is defined as a progressive infection of the bone that results in inflammatory destruction, bone necrosis, and new bone formation and may progress to a chronic and persistent state. It can be divided into acute haematogenous, subacute, post-traumatic, and chronic osteomyelitis [1]. Chronic OM is one of the most severe complications in the orthopaedic field [2]. Bacteria usually cause this bone infection, mainly *Staphylococcus aureus*, in approximately call for 75 % of cases [3].

Chronic osteomyelitis is challenging to treat because it is often associated with necrosis of bone and poor vascular perfusion accompanied by an infection of the surrounding tissues [4]. The treatment of osteomyelitis mainly involves operative debridement, surgical removal of necrotic tissue, and antibiotic therapy. Antibiotics must be administered at a concentration many times higher than the minimum bactericidal concentration to eradicate bacteria encased in such a biofilm [5]. The high dose and a prolonged course of treatment can lead to systemic toxicity of the antibiotic. Besides, the prolonged use of parenteral antibiotics, with multiple surgical debridements, is often required for effective therapy [6]. Repeated failures of these therapies often result in the removal of the orthopaedic implants, which is costly and traumatic to the patient.

Since delivering antibiotics to the target site at a sufficiently high concentration by the intravenous route is impractical, a drug delivery system (DDS) cement beads technology is developed for delivering the antibiotics localised [7]. The biodegradable and biocompatible DDS delivery vehicles seem to be a promising alternative [8]. This technology did not require a second surgery to remove the implant. Besides, it allows for a wider variety of antimicrobials in the carriers.

The application of impregnated biomaterials with gentamicin is well-established as a treatment for OM [9]. The factors include effective infection suppression at an early stage, suitable for bone generation, and a noticeable reduction of the recurrent infection incidence. This technology's most widely used biomaterial is poly (methyl methacrylate) (PMMA) mixed with antibiotics [7,8,10]. This technology offers localized high concentrations of antibiotics without causing hypersensitivity reactions and is the gold standard of local delivery as OM treatment [11]. However, non-biodegradable beads require removal through a second surgery. Prolonged-release of subinhibitory concentration of antibiotics is worrisome in the clinical application of antibiotic-loaded bone cement, as it stimulates the introduction of gentamicin-resistant strains. This risk has paved the way for exposure to resistant strains open to the importance of developing biodegradable antibiotic-loaded beads as an antibiotic delivery.

Therefore, gentamicin impregnated with biomaterials beads is being developed to overcome the disadvantage of methyl methacrylate. This technology provides a slow residual release of antibiotics for a definite period, and the biodegradability of the carrier beads avoids the need for a second surgery for the removal after therapy [12-14]. Therefore, in this study, we investigated the potential of impregnated biomaterials with gentamicin against infection through an in vivo experimental setting.

Materials and Methods

Bacterial Strain

The *Staphylococcus aureus* Rosenbach strain American Type Culture Collection (ATCC) 25923 (Culti-Loops^{TM}, OXOID) was used in this study. These strains were directly streaked in nutrient agar (Merck, Germany) and incubated at 37 °C for 24 hours in a CO_2 incubator (Innova CO-170, New Brunswick Scientific) to obtain a single colony from the direct plating inoculum. Then, a single colony was streaked from agar and transferred to 5 ml of nutrient broth (Merck, Germany). The inoculum was incubated at 37 °C for 24 hours for culture and was prepared for the following downstream work.

Preparation of Treatment Impregnated Biomaterials with Gentamicin

Biomaterials Hydroxyapatite (HA) (GranuMas®, Granulab Malaysia Sdn. Bhd.) and Calcium sulphate ($CaSO_4$) (MIIG®X3, Wright Medical EMEA, Amsterdam, NLD) were used in this study (patent pending without prejudice). The biomaterials (were mixed with its solution), gentamicin antibiotic (40 mg/ml), and agar-agar (solution) were mixed vigorously until the mixture became homogenous liquid and waited until it became paste form. They were then moulded to form beads with a size of approximately 3 x 4 mm cylindrical of each bead. Each bead contains gentamicin with a dose of approximately 9 mg. The beads were then sterilized under ultraviolet light. All procedures were conducted under the aseptic technique.

Animal Model

All surgical procedures in this study were performed at the Advanced Orthopaedic Research Laboratory (ORL), Department of Orthopaedics, Traumatology, and Rehabilitation, Kulliyyah of Medicine, International Islamic University Malaysia (IIUM), Kuantan, Pahang. The management system of ORL in following with the ISO/IEC 17025:2005 standards. This study was approved by the International Islamic University Malaysia's Institutional Animal Care and Use Committee (IACUC-IIUM), with approval letter reference number: IIUM/504/14/2/IACUC. This study used 36 New Zealand White Rabbit (NZWR) Oryctolagus cuniculus weighing 2.5 to 4.2 kg. The age was older than six months. The age and weight were chosen based on the rabbit's maturity and size to ensure better tolerance for surgery.

Experimental Design

A total of 36 rabbits were randomly divided into three groups; the $CaSO_4$ group treatment (consisting of 16 rabbits), the HA group treatment (consisting of 16 rabbits), and the sham group consisting of 4 rabbits (without treatment). The gentamicin beads impregnated with biomaterial ($CaSO_4$ and HA) were further subdivided into four groups for 3, 6, 12, and 26 weeks for different observation periods.

Anaesthesia was administered by well-trained personnel. Ketamine (Ketapex, Apex Laboratories Pty Ltd., Australia), Tilatamine / Zolezepam (Zoletil® 50, Virbac Laboratories, Carros, France), and Xylazine were used to sedate rabbits (Ilium Xylazil-20, Troy Laboratories Pty Ltd, Australia). The mixture was injected intramuscularly at 0.2 ml/kg body weight for induction and maintained intravenously at 0.1 ml/kg via the ear's marginal veins.

The level of consciousness in the animals was monitored by examining the changes of heart and respiratory rate, jaw tone (resistance of opening the mouth), pedal withdrawal reflex, and rabbit body temperature to prevent hypothermia. An additional anaesthesia regime was given to maintain sedation during the surgery.

Surgical Technique

Development of Osteomyelitis

This study was divided into two (2) sections. The first section of this study focused on the induce of osteomyelitis. *Staphylococcus aureus* ATCC 25923 was used to develop osteomyelitis. After taking a preoperative lateral plain radiograph of the interested site, all groups underwent the first operation to create osteomyelitis at the right distal femur. Swab culture and sensitivity tests were taken before bacteria inoculation. After 3 weeks of operation, the lateral plain radiograph of the rabbit femur was taken to check for evidence of osteomyelitis.

Treatment Implantation

The second part of this study was developing a treatment (impregnated of biomaterials with gentamicin). Two types of treatment were developed; impregnated calcium sulphate with gentamicin (later referred to as $CaSO_4$ group) and impregnated HA with gentamicin (later referred to as HA group). After the radiograph procedure, all the rabbits underwent the second operation for debridement and implanted treatment according to the group. Before debridement, the swab culture was taken from the operated site in all groups.

Sample Harvesting

All animals were sacrificed according to the intervals at 3 weeks, 6 weeks, 12 weeks, and 26 weeks using an overdose (1 ml/kg of rabbit of 100 mg/ml ketamine hydrochloride drugs intraveneous injection. The bone sample was harvested under the aseptic technique by making an incision of the skin over the leg. The rabbit fascia was incised with mayo scissors. The muscle layer was cut to expose the bone by using a scalpel. The bone sample was fixed in a 10% natural buffered formalin solution. All rabbits were euthanized for assessments (microbial analysis, radiographic evaluation, micro-CT scan constructs, gross observation, and histology interpretation) according to the intervals selected.

Microscopic Evaluation

Post-mortem of Microbial Analysis

Following sacrifice, the distal femur was harvested aseptically. Swabs were taken from the surrounding implanted area. The post-mortem of microbial analysis was performed to evaluate the treatment's effectiveness. Swab inoculation was smeared on sterile nutrient agar media and incubated at 37 °C for 24 hours for aerobic bacterial culture. Gram staining, catalase, and coagulase tests were performed to confirm the type of organisms.

Post-mortem of Imaging Evaluation

After the animal was euthanized, the distal femur was taken for imaging evaluation. Lateral, anterior-posterior plain radiographs and ex vivo micro-CT imaging of the bone and implant area were performed using Bruker® Skyscan 1176, Belgium. The samples were scanned using Al 1 mm filter and 18 µm pixel resolutions. The excised distal femur was taken in all groups to check for result interpretation.

Histology Interpretation

The harvested femur was sliced in the coronal plane into a few pieces at least 0.5 cm thick for each section using the EXAKT cutter machine. Initially, the samples were fixed in 10 % Neutral Buffered Formalin (NBF). Then, the samples were dehydrated in a graded series of alcohol and infiltrated with a series of methyl methacrylate (Technovit® 7200, Heraeus Kulzer Co., Germany). The samples were then embedded in methyl methacrylate (Technovit® 7200, Heraeus Kulzer Co., Germany), sectioned at a thickness of 80 µm (EXAKT Apparatebau systems, Norderstedt, Germany), and stained with Masson Goldner Trichrome staining. All sections were visualized using a motorized transmitted light research motorized microscope (Nikon Eclipse Ni, Japan) for histological interpretation.

Results and Discussion

Post-mortem of Microbial Analysis

The Microbial Assessments results show the microbial study results from 3 weeks to 26 weeks after inoculation. The results show that by 26 weeks, no apparent bacteria growth was noted from both groups (Figures 1(D) and 2(D)). After 3 weeks of the inoculation of *Staphylococcus aureus*, all subjects showed growth of the bacteria (Figure 1(A) and 2(A)), which is comparable to the sham group (Figure 3). The success rate of osteomyelitis in the rabbit's femur was 100 % through in-house technique [15]. Microbial analysis showed that after 26 weeks, there was no *Staphylococcus aureus* isolated from both $CaSO_4$ and HA groups, respectively. Similar findings found in commercial bone cement product containing calcium sulphate and hydroxyapatite was successfully incorporated as a carrier vehicle, with all heat-sensitive and heat-stable antibiotics tested, releasing the antibiotics at a sufficient level for treating specific bacteria [7-9]. The incorporation of these two biomaterials created in vitro significant zones of inhibition, hence showing susceptibility against *Staphylococcus aureus* species, which holds immense promise in treating osteomyelitis in situ. Gentamicin with bone cement (PMMA) is already used for joint replacement surgery [9]. It is widely used in total knee replacement and total hip replacement surgery [16]. It is also used in revision joint replacement and infected joint replacement. The results from this study may open the possibility of using impregnated biomaterial in future clinical and joint replacement surgery.

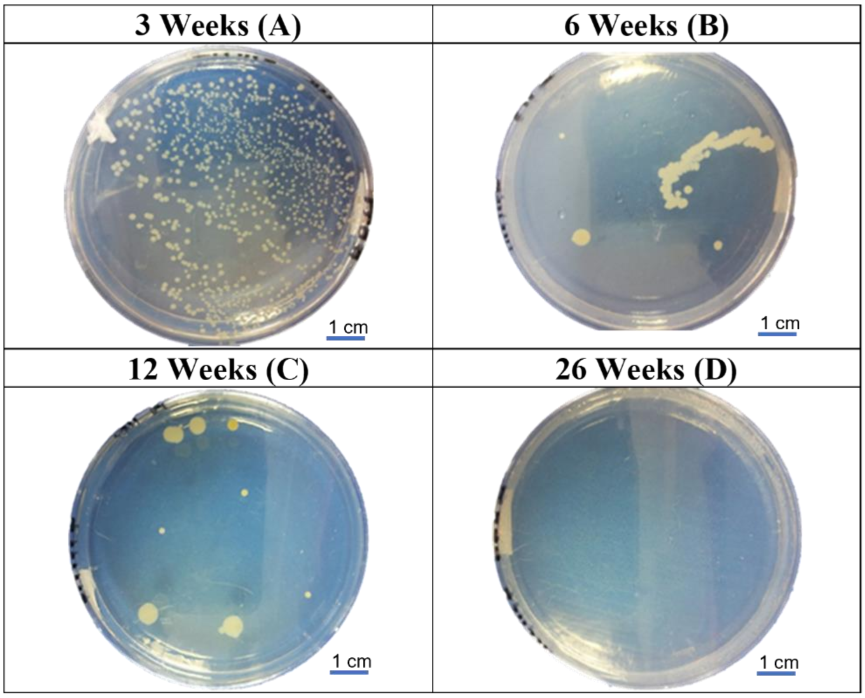

Figure 1: The microbial study shows the outcome from 3 to 26 weeks of post-treatment gentamicin beads impregnated with HA.

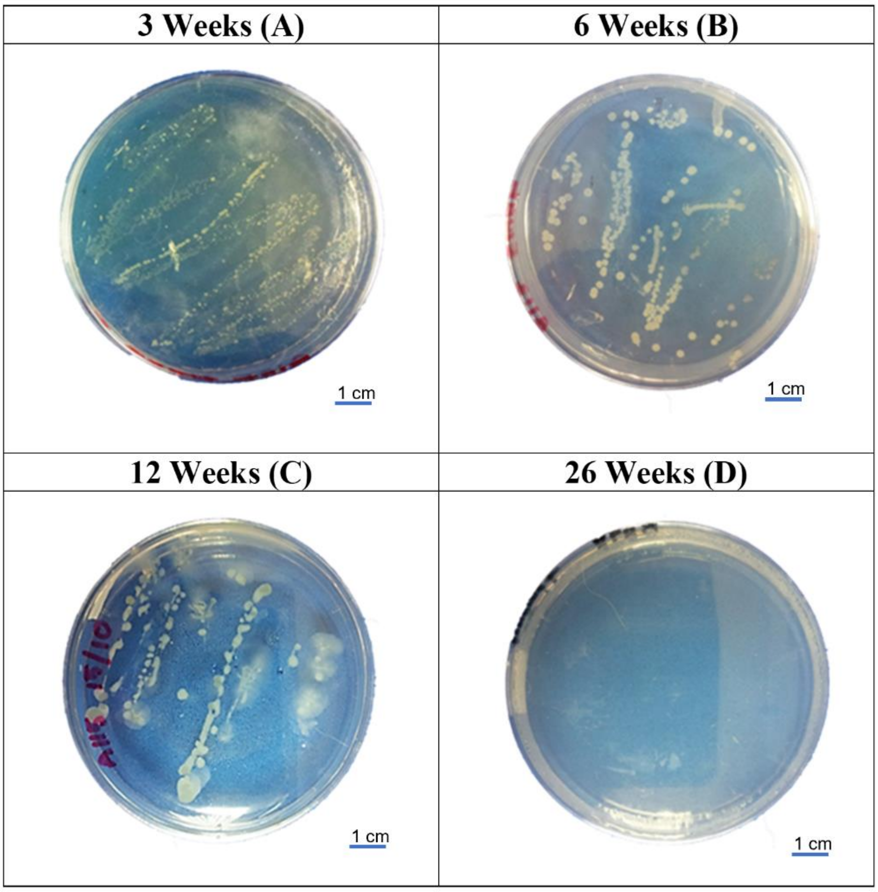

Figure 2: The microbial study shows the outcome from 3 to 26 weeks of post-treatment gentamicin beads impregnated with $CaSO_4$.

Figure 3: The diagram above shows that *Staphylococcus aureu*s growth was noted after 3 weeks of inoculation in the Sham group.

Post-mortem of Imaging Evaluation

In 3 weeks of post-treatment assessment in Micro-CT 2D images, the drilled hole was still present in both groups (Figures 4(A) and 5(A)). The biomaterials were seen at the defective hole site. Periosteum elevation was observed in both groups. Meanwhile, 6 weeks of treatment shows that the drilled hole was still present (Figures 4(B) and 5(B)). HA material was in situ, while $CaSO_4$ material was minimally present. There was an elevation in bony changes with multiple lacunae at the cortical bone area. The Haversian canal enlargement was noted in both groups.

In addition, 12 weeks of treatment shows that the drilled hole in the group treated with gentamicin impregnated HA is in situ (Figure 4(C)). As for the gentamicin impregnated $CaSO_4$ group, the drilled hole was united, and the biomaterial was absent (Figure 5(C)). The Haversian canal enlargement was in situ. Bony changes with multiple lacunae were seen at the cortical bone in both groups. However, 26 weeks shows that the drilled hole united in both groups. No biomaterials were seen in the group treated with gentamicin impregnated $CaSO_4$ (Figure 5(D)). HA was still present in situ since it would take more time to dissolve into the bone (Figure 5(D)). Both groups showed less cortical bone lacunae. After treatment (as in radiology and histology results), the bone structure and architecture improved at 12 weeks. After treatment, both groups showed the formation of new trabecular, and the affected distal femur was restored to its normal anatomical structure. This is due to the new bone that formed infection, both the formation and resorption of the bone were affected by the infection [2,3].

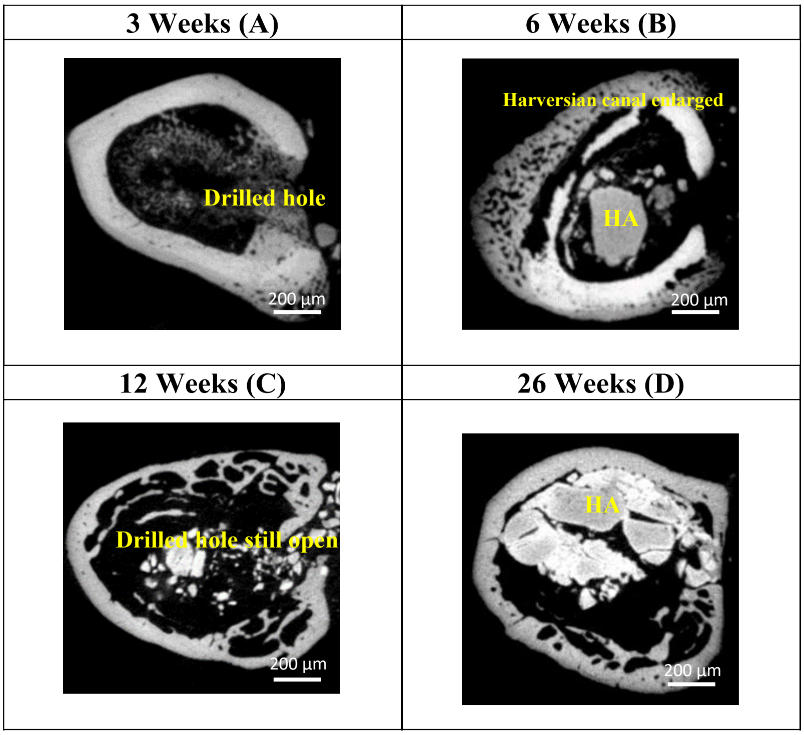

Figure 4: Micro-CT 2D images from 3 to 26 weeks post-treatment of gentamicin beads impregnated with HA.

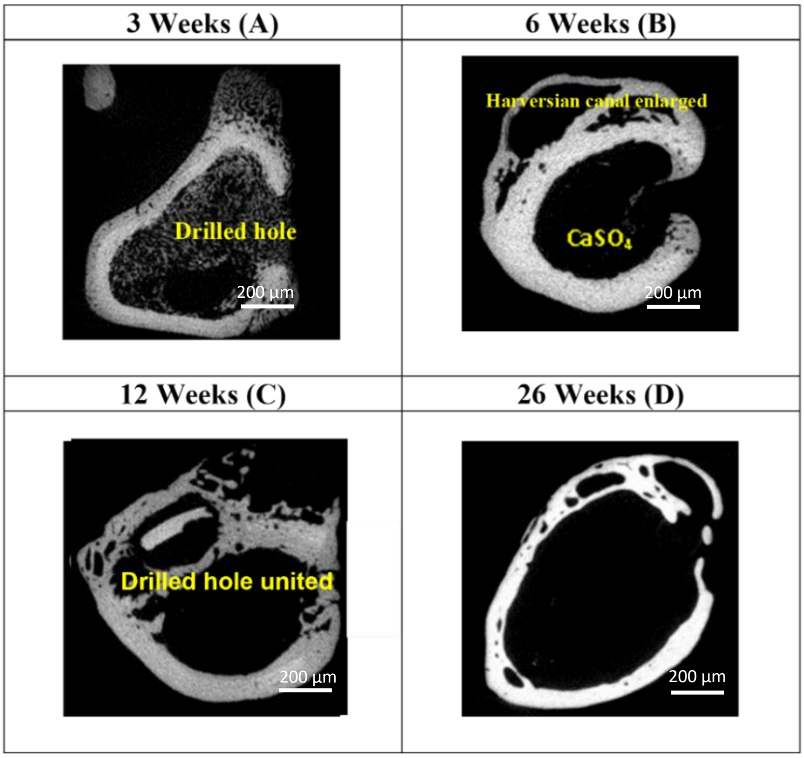

Figure 5: Micro-CT 2D images from 3 to 26 weeks post-treatment of gentamicin beads impregnated with $CaSO_4$

Histology Interpretation

After 3 weeks of post-treatment assessment, the histology findings show that both groups present cortical bone swelling, periosteal reaction, and the defect state (Figures 6(A) and 7(A)). Meanwhile, at 6 weeks of post-treatment, the histology findings show multiple Haversian canal enlargement and periosteum reaction was noted in both groups, as illustrated in (Figures 6(B) and 7(B)). The drilled hole was present on the union of the cortical bone in both groups. In addition, at 12 weeks of post-treatment, it shows the presence of biomaterial in the gentamicin impregnated with HA groups (Figure 6(C)). In contrast, the absence of biomaterial in the gentamicin impregnated with $CaSO_4$ group was noted (Figure 7(C)). No periosteal reaction was noted, the Haversian canal enlargement was present in both groups, and the drilled hole was closed in both groups. The biomaterials did not hinder the formation of new bone growth into the infected site and did not cause chronic inflammation as it is biodegradable material [10-12]. Both groups also formed osseous bridging over the defect to enhance bone healing.

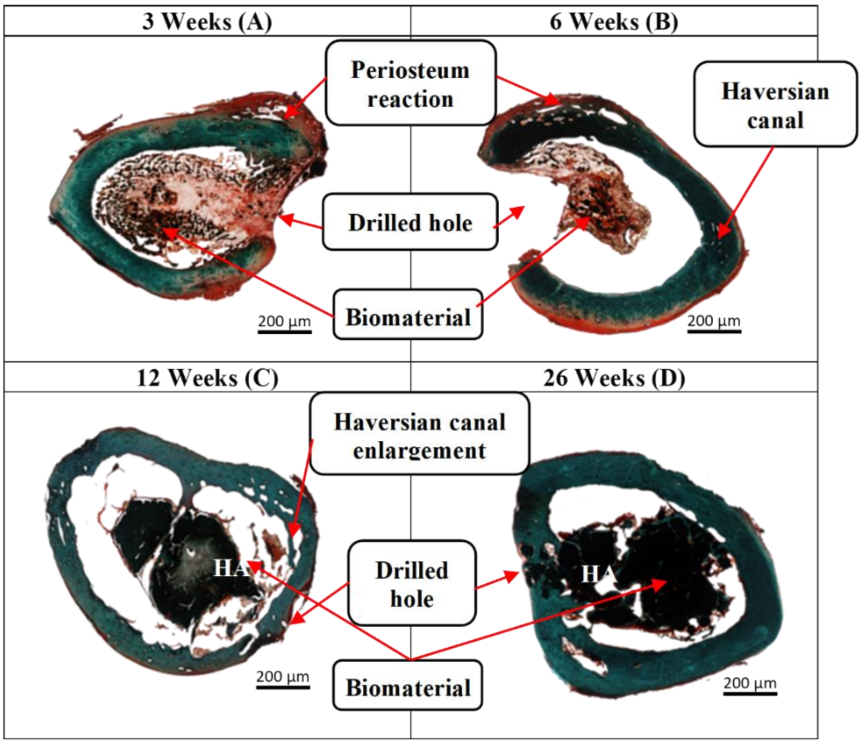

Figure 6: Histology images from 3 to 26 weeks post-treatment of gentamicin beads impregnated with HA (Masson Goldner Trichrome stain, original magnification 40x)

As for 26 weeks of post-treatment, the presence of biomaterial in the gentamicin impregnated with the HA group was noted (Figure 6(D)). However, the absence of biomaterial in the gentamicin impregnated with enlargement of the $CaSO_4$ group was noticeable in this evaluation (Figure 7(D)). Compared to the HA group, the haversian canal was noted in the gentamicin impregnated with $CaSO_4$. The bone defect was closed in both groups. $CaSO_4$ biomaterials were wholly resorbed, but as for HA, it was still present in situ. This phenomenon mimics the phase of bone mineralization and the absorption rate of bone formation even though the absorption from the two biomaterials is different [2]. In addition, both biomaterials also act as a space filler, improving the morphological contour of the bone, thus restoring the normal anatomical and structural of the bone [10,11]. These results supported that both gentamycin impregnated with $CaSO_4$ and hydroxyapatite eradicate bacterial infection of the bone and help regenerate new bone.

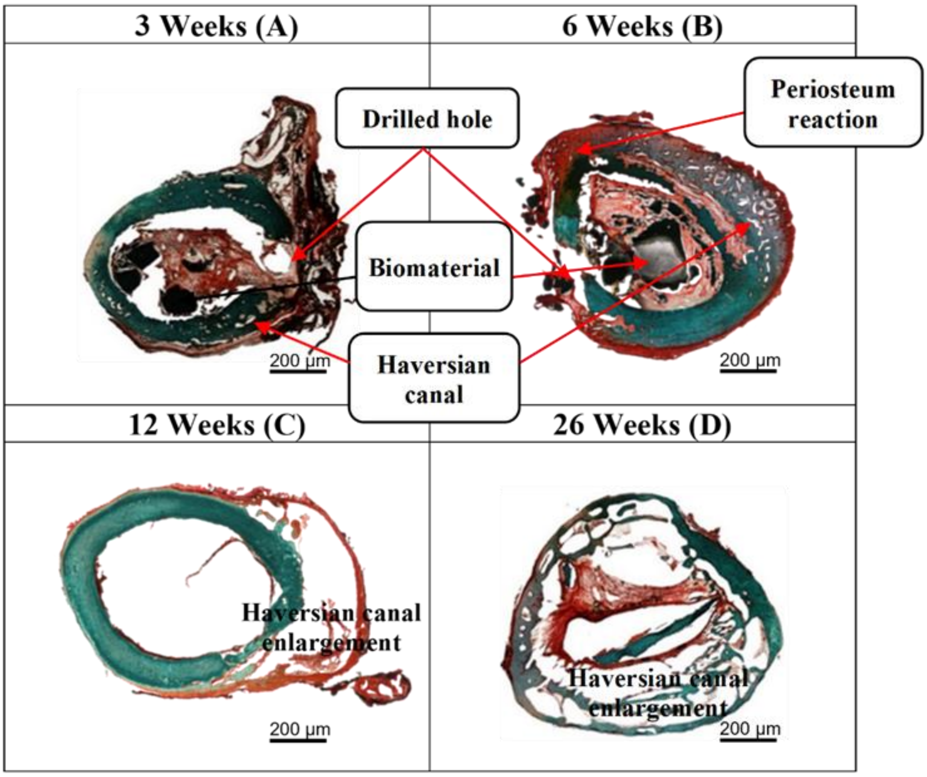

Figure 7: Histology images from 3 to 26 weeks post-treatment of gentamicin beads impregnated with $CaSO_4$ (Masson Goldner Trichrome stain, original magnification 40X)

Conclusions

The study has shown a good outcome and the benefit of using gentamicin impregnated with biomaterials ($CaSO_4$ and HA). The results show the potential of using gentamicin impregnated with biomaterials in a clinical setup. Both groups showed bone generation and healing at the end of the study interval (26 weeks). In conclusion, the study shows that gentamicin impregnated with HA can treat osteomyelitis of the femurs of NZWRs as they give good results. This is supported by the results obtained from the microbiological results, micro-CT analysis, and histological results. These findings may pave the opportunity for clinical trials and applications.

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Author Contributions

All authors contributed to the paper's data analysis, drafting, and revisions, agreeing to accept responsibility for all aspects of the work.

Disclosure of Conflict of Interest

The authors have no disclosures to declare.

Compliance with Ethical Standards

The work is compliant with ethical standards

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