

RESEARCH ARTICLE

Dose-dependent effects of local silver nanoparticle application on fracture healing and antioxidant responses in a rat femoral osteotomy model



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ABSTRACT

Background and Aim: Silver nanoparticles (AgNPs) have potential applications in bone regeneration; however, the concentration-dependent effects of their direct local administration at fracture sites remain insufficiently characterized. This study evaluated the effects of a single local application of AgNPs at different concentrations on fracture healing in a stabilized rat femoral osteotomy model and assessed associated changes in antioxidant enzyme activity.

Materials and Methods: Eighty healthy male albino rats, aged 5–6 months and weighing 300–350 g, were allocated equally to four groups ($n = 20/\text{group}$): control and AgNP treatments at 2.5, 5, and 10 $\mu\text{g/mL}$. Following standardized femoral osteotomy and intramedullary fixation, 1 mL of normal saline or the assigned AgNP suspension was administered once locally at the osteotomy site. Fracture healing was evaluated on postoperative days 7, 14, 21, and 30 using radiographic callus measurements, histological assessment, and histomorphometric quantification of osteoblasts, osteocytes, and osteoclasts. Catalase (CAT) and superoxide dismutase (SOD) activities were assessed on days 7 and 30.

Results: Local AgNP administration showed concentration- and time-dependent differences in fracture healing. On day 30, callus diameters were 5.28 ± 0.09 , 6.20 ± 0.11 , 7.01 ± 0.15 , and 8.56 ± 0.05 mm in the control, 2.5, 5, and 10 $\mu\text{g/mL}$ groups, respectively ($p < 0.05$). Histomorphometry demonstrated corresponding changes in bone-cell dynamics. On day 30, osteocyte counts were 3.60 ± 0.66 , 8.53 ± 0.69 , 10.14 ± 0.66 , and 12.03 ± 1.39 cells/high-power field, respectively, whereas osteoblast counts were 5.53 ± 0.69 , 7.87 ± 0.58 , 10.73 ± 0.80 , and 10.80 ± 0.84 cells/high-power field, respectively. CAT activity increased, whereas SOD activity generally decreased with increasing AgNP concentration.

Conclusion: A single local AgNP application was associated with concentration-dependent increases in radiographic callus formation and changes in histomorphometric indices of fracture repair, with the most pronounced responses generally observed at 10 $\mu\text{g/mL}$. AgNP exposure also altered antioxidant enzyme concentrations. However, these findings do not establish enhanced biomechanical strength or a specific mechanism of action. Further biomechanical, molecular, pharmacokinetic, and toxicological studies are required to establish functional efficacy and safety.

Keywords: antioxidant enzymes, bone regeneration, fracture healing, histomorphometry, local administration, rat femoral osteotomy, silver nanoparticles, tissue remodeling.

INTRODUCTION

Fracture healing is a complex biological process involving sequential and overlapping phases of inflammation, soft-callus formation, mineralization, hard callus development, and subsequent bone remodeling. Successful fracture repair requires coordinated interactions among inflammatory responses, cellular proliferation and differentiation, extracellular matrix formation, vascularization, tissue regeneration, and bone remodeling. Although most uncomplicated fractures heal satisfactorily, delayed healing and infection-related complications remain important challenges in orthopedic surgery. Consequently, attention has increasingly focused on local therapeutic strategies that may support bone repair while minimizing unnecessary systemic exposure.

Silver nanoparticles (AgNPs) have attracted considerable interest in orthopedic and regenerative medicine because of their physicochemical and antibacterial properties and their reported biological effects on cells involved in bone regeneration. Researchers have investigated AgNPs in hydrogels, scaffolds, implants, and other biomaterial-based systems, primarily because they can reduce bacterial colonization while exerting biological effects relevant to bone repair. Zhang *et al.* [1] reported that AgNPs enhanced fracture callus formation and

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fracture gap closure and promoted mesenchymal stem-cell proliferation and osteogenic differentiation in a mouse femoral fracture model. Similarly, Sabry *et al.* [2] demonstrated the osteogenic potential of AgNPs in a critical-sized mandibular bone-defect model in rats. Other studies have investigated the effects of AgNP-based orthopedic biomaterials on osteogenic cells, including mesenchymal stem cells and osteoblasts [3, 4].

However, the biological response to AgNPs depends on several factors, including particle size, concentration, surface characteristics, stabilizing or coating agents, formulation, duration of exposure, and route of administration. Consequently, findings obtained using one AgNP formulation or delivery system may not be directly applicable to another [3, 5]. Reviews on AgNP applications in orthopedics and bone tissue engineering have further emphasized the predominantly preclinical nature of the available evidence and the need for additional investigations into efficacy, safety, dose dependency, biocompatibility, and appropriate delivery strategies [5, 6]. These considerations are particularly important because increasing AgNP concentrations may adversely affect bone-forming cells under certain experimental conditions [3, 4].

Despite growing interest in AgNPs for bone regeneration, much of the available evidence comes from AgNPs incorporated into scaffolds, coatings, hydrogels, bone substitutes, or other composite delivery systems rather than from a single, directly administered local treatment at a surgically created fracture site. These delivery systems may alter nanoparticle availability and tissue exposure, making it difficult to extrapolate findings to direct local administration. Furthermore, the concentration-dependent response to locally administered AgNPs during fracture repair remains insufficiently characterized. Differences in particle properties, concentrations, formulations, and delivery approaches across previous studies further limit direct comparison and prevent reliable prediction of the fracture healing response to a defined local AgNP dose [3–6]. Therefore, a controlled evaluation of multiple concentrations of a physicochemically characterized AgNP preparation within the same mechanically stabilized fracture model is needed to clarify whether increasing local exposure is associated with measurable differences in fracture healing progression and antioxidant responses.

In the present study, AgNPs were produced using a chemical reduction method and characterized before biological application. Transmission electron microscopy demonstrated a primary particle size of approximately 20 nm, whereas dynamic light scattering indicated a Z-average hydrodynamic diameter of 33.13 nm. Concentrations of 2.5, 5, and 10 µg/mL were selected as an experimental dose range to evaluate concentration-dependent responses rather than as established therapeutic concentrations. A single 1-mL application therefore delivered 2.5, 5, or 10 µg of AgNPs directly to the fracture site.

This study evaluated the dose-dependent effects of a single local administration of AgNPs at 2.5, 5, and 10 µg/mL on fracture healing in a mechanically stabilized rat femoral osteotomy model. Fracture repair was evaluated through serial radiographic assessment of callus formation and histological and histomorphometric evaluation of osteoblast, osteocyte, and osteoclast populations. In addition, local catalase (CAT) and superoxide dismutase (SOD) activities were measured to characterize antioxidant enzyme responses associated with AgNP exposure. We hypothesized that increasing local AgNP concentrations would be associated with concentration-dependent differences in radiographic callus formation and histomorphometric indices of fracture repair, accompanied by measurable changes in local CAT and SOD activities.

MATERIALS AND METHODS

Ethical approval

The study protocol was reviewed and approved by the Ethics and Scientific Committee of the College of Veterinary Medicine, University of Sulaimani (approval no. VMUS.EC.DOC.14-2024; December 2024). All experimental procedures were conducted in accordance with the institutional guidelines for the care and use of laboratory animals. The experimental protocol incorporated the principles of replacement, reduction, and refinement, particularly reduction, by limiting the number of animals to that required for radiographic, biochemical, histological, and histomorphometric assessments. Animals were monitored daily after surgery for general clinical condition, food and water intake, mobility, surgical wound appearance, signs of infection, and abnormal pain-related behavior.

Study period and location

The study was conducted from June 1, 2025, to June 1, 2026, at the Veterinary Teaching Hospital, College of Veterinary Medicine, University of Sulaimani, Kurdistan Region, Iraq. Surgical procedures, postoperative monitoring, radiographic examinations, and associated experimental assessments were performed within the facilities of the College of Veterinary Medicine.

Study design

This controlled experimental study included 80 healthy male albino rats aged 5–6 months and weighing 300–350 g. The animals were allocated equally to four groups ($n = 20/\text{group}$): a control group and three AgNP-treated groups receiving concentrations of 2.5, 5, or 10 $\mu\text{g}/\text{mL}$. Twelve animals from each group were distributed among four postoperative assessment points (days 7, 14, 21, and 30), with three animals per group at each time point ($n = 3$) used for radiographic, histological, and histomorphometric evaluations. The remaining eight animals in each group were allocated to biochemical assessment, with four animals evaluated on postoperative day 7 and four on day 30 ($n = 4/\text{time point}$).

The sample size was established in accordance with the approved animal-use protocol, considering the requirements for radiographic, biochemical, histological, and histomorphometric assessments while adhering to the principles of replacement, reduction, and refinement. An a priori statistical power analysis was not performed.

AgNPs preparation and physicochemical characterization

AgNPs were synthesized in the laboratory using a chemical reduction method. Silver nitrate (AgNO_3 ; Sigma-Aldrich, St. Louis, MO, USA, Catalog No. 204390) was used as the silver precursor. AgNO_3 was dissolved in distilled water, and Ag^+ ions were reduced to elemental metallic silver using sodium borohydride (NaBH_4 ; Sigma-Aldrich, Catalog No. 452882)) as the reducing agent. Polyvinylpyrrolidone (Sigma-Aldrich, Catalog No. PVP40, average molecular weight $\sim 40,000$) was used as a stabilizing agent to minimize nanoparticle aggregation and maintain a stable aqueous suspension. Because AgNO_3 contains approximately 63.5% elemental silver by weight, the amount of precursor was calculated according to the required quantity of elemental silver.

The synthesized AgNPs were characterized using transmission electron microscopy, dynamic light scattering, zeta potential analysis, and X-ray diffraction. Transmission electron microscopy demonstrated predominantly spherical nanoparticles with a primary particle size of approximately 20 nm. Dynamic light scattering analysis of the aqueous suspension at 25°C demonstrated a Z-average hydrodynamic diameter of 33.13 nm and a polydispersity index of 0.419. The difference between the primary particle size determined by transmission electron microscopy and the larger hydrodynamic diameter determined by dynamic light scattering reflects particle behavior in aqueous suspension, including the surrounding hydration layer and dispersion characteristics. The measured zeta potential was -14.5 mV. X-ray diffraction analysis demonstrated characteristic diffraction reflections consistent with the face-centered cubic crystalline structure of metallic silver.

For animal administration, the synthesized AgNP suspension was prepared at concentrations of 2.5, 5, and 10 $\mu\text{g}/\text{mL}$. Because each animal received 1 mL of the designated suspension, the corresponding amounts administered were 2.5, 5, and 10 μg of AgNPs per application, respectively. Suspensions were prepared aseptically and ultrasonicated immediately before administration to promote uniform nanoparticle dispersion. Under the laboratory conditions used for evaluation, Ag^+ release from the AgNP preparation was detected at 0.1–0.5 ppm, confirming that a measurable fraction of Ag^+ ions was released. The independent contribution of released Ag^+ ions to the observed biological responses was not evaluated.

Preoperative management and anesthetic technique

Before surgery, each animal was weighed and clinically examined to confirm its general health status. Animals were not fasted before surgery. General anesthesia was induced using an intraperitoneally administered ketamine–xylazine combination. The anesthetic mixture consisted of 0.7 mL ketamine (100 mg/mL injectable solution; Alfasan Nederland B.V., Woerden, Netherlands (Product: Ketamine 10%, 100 mg/mL solution). Alternatively: Richter Pharma, Wels, Austria.) and 0.3 mL xylazine (20 mg/mL injectable solution; Alfasan Nederland B.V., Woerden, Netherlands). Anesthesia was maintained throughout the surgical procedure without additional anesthetic administration. Body temperature, heart rate, and respiratory rate were monitored during surgery and remained within acceptable ranges under the experimental conditions. Anesthetic and perioperative management was performed with consideration of established recommendations for anesthesia and perioperative care in laboratory rodents [7].

Surgical procedure, femoral osteotomy model, and fixation

Each animal was positioned in left lateral recumbency to expose the right hind limb. The lateral aspect of the right femoral region was clipped, shaved, and aseptically prepared with povidone-iodine (Dermosept Company, Kim-Pa ilaç San. Tic. Ltd. Şti. (Dermosept), Istanbul, Turkey [Typically supplied as 10% povidone-iodine antiseptic solution]). The surgical field was then draped using sterile material. A 2–3-cm skin incision was made over the

femur, followed by dissection through the subcutaneous tissue and fascia lata. The semimembranosus and vastus lateralis muscles were separated by blunt dissection to expose the femoral shaft. The periosteum was incised to expose the cortical surface.

Under aseptic conditions, a standardized complete transverse mid-diaphyseal femoral osteotomy with an approximately 0.5-mm gap was created using a fine electric saw (KUGEL medical company, KUGEL medical GmbH & Co. KG, Regensburg, Germany) equipped with a 10-mm-wide, 0.5-mm-thick blade. A 0.5-mm spacer was used during osteotomy to standardize the fracture gap among animals. After osteotomy, we checked and measured the gap with a surgical vernier caliper with 0.5-mm resolution, which provided sufficient accuracy to verify the 0.5-mm osteotomy gap.

The osteotomy was stabilized with a 1-mm stainless-steel intramedullary pin (Fine Science Tools (FST), Foster City, CA, USA), selected based on the approximate diameter of the femoral medullary canal. After reduction, the pin was inserted retrogradely through the medullary cavity of the proximal fragment until it emerged proximally in the intertrochanteric region, then redirected into the distal fragment. This procedure maintained alignment of the osteotomized femoral segments and provided internal stabilization. Standardized surgical procedures, animal allocation, and postoperative assessment are important methodological considerations in experimental rat surgery [7].

Experimental groups and AgNP administration

Following osteotomy and stabilization, animals received the designated treatment locally at the osteotomy site (Figure 1).

Group A (control; n = 20) received a single local application of 1 mL normal saline.

Group B (n = 20) received a single local application of 1 mL AgNP suspension at 2.5 µg/mL (2.5 µg AgNPs per animal).

Group C (n = 20) received a single local application of 1 mL AgNP suspension at 5 µg/mL (5 µg of AgNPs per animal).

Group D (n = 20) received a single local application of 1 mL AgNP suspension at 10 µg/mL (10 µg of AgNPs per animal).

After administration, the muscle layer was closed with 3-0 absorbable Vicryl (Ethicon Inc., Somerville, NJ, USA/Product Code: W9113 or VCP304H), and the fascia and skin were closed with 3-0 nonabsorbable silk (Ethicon Inc., Somerville, NJ, USA, Product Code: W502).

Postoperative management

After surgery, animals recovered under warm conditions before being returned to their cages. Food and water were provided *ad libitum*. Animals were maintained under cage-rest conditions with unrestricted movement within their cages throughout the postoperative period.

General clinical condition, food and water intake, mobility, surgical wound appearance, signs of infection, and abnormal pain-related behavior were assessed daily. No postoperative analgesics or antibiotics were administered. During postoperative monitoring, no serious illness, wound dehiscence, persistent abnormal pain-related behavior, severe infection, marked weight loss, or difficulty consuming food or water was observed.

Current recommendations for laboratory rodents emphasize appropriate anesthesia, analgesia, perioperative monitoring, and postoperative care as important components of experimental surgical protocols [7]. Because perioperative and postoperative interventions can influence physiological and oxidative responses in laboratory rodents, these factors were considered when interpreting fracture healing outcomes. Previous experimental evidence indicates that postoperative analgesic treatment can influence oxidative stress parameters in rats, so postoperative analgesics were purposely removed from the protocol to avoid potential confounding pharmacological effects on bone healing, inflammation, or osteogenesis, which would otherwise interfere with the evaluation of the tested nanomaterials [8]. Standardized animal allocation, anesthesia, surgical procedures, and postoperative assessment have similarly been emphasized in experimental rat surgery [9].

Radiographic imaging and ImageJ analysis

Radiographic examinations were performed at the Veterinary Teaching Hospital on postoperative days 7, 14, 21, and 30 to evaluate fracture healing progression and quantify callus formation. Callus measurements were performed using ImageJ software (version 1.49, National Institutes of Health, Bethesda, Maryland, USA,), with the same measurement procedure applied to all experimental groups and assessment points. Callus diameter was

used as a quantitative radiographic indicator of callus formation in accordance with previously published methodology [10].

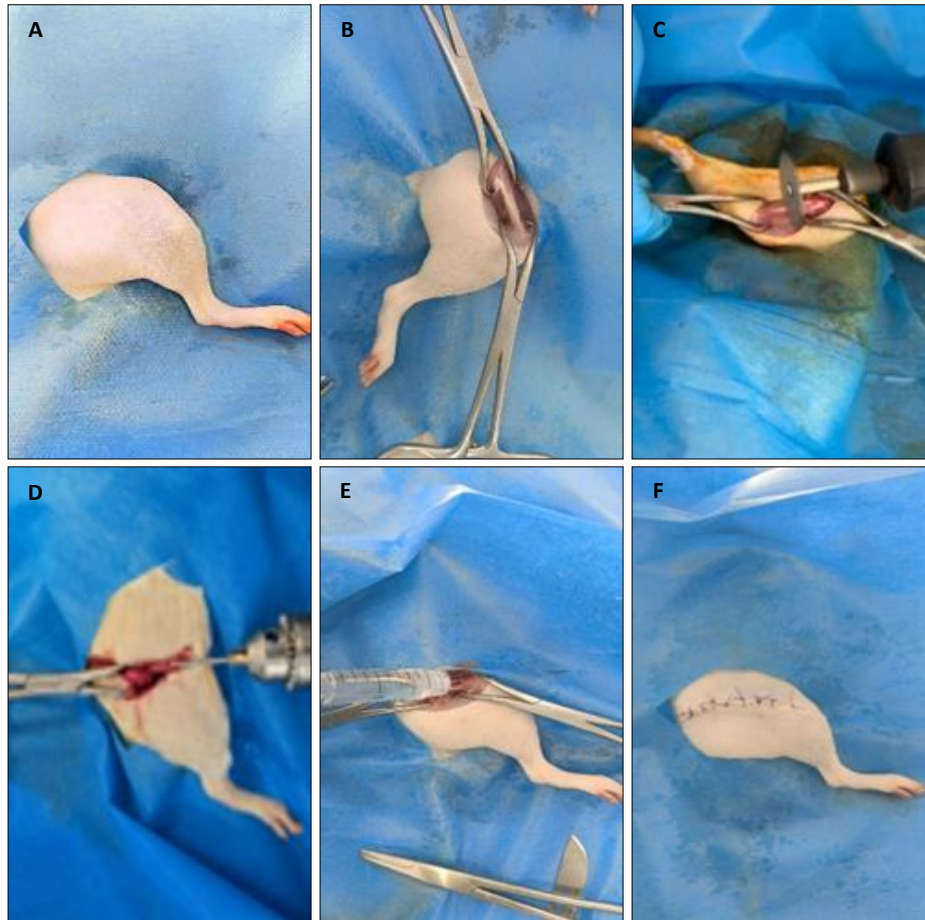


Figure 1: Surgical creation and treatment of the stabilized femoral osteotomy model in rats: (a) preparation of the skin over the femoral region; (b) exposure of the femur; (c) creation of the transverse femoral osteotomy using an electric bone saw; (d) insertion of the intramedullary pin using a pin chuck; (e) local application of the silver nanoparticle suspension at the osteotomy site; (f) closure of the surgical incision.

Each radiograph was calibrated before measurement using the radiographic ruler incorporated into the image. A line corresponding to a known distance on the ruler was drawn using the straight-line selection tool, and the corresponding distance was entered using the Analyze Set Scale function to convert pixel measurements to actual dimensions. The same anatomical region at the osteotomy site was evaluated on each radiograph.

The maximum transverse callus diameter and underlying bone diameter at the osteotomy site were measured using the straight-line tool. Callus thickness extending beyond the original cortical margin was calculated by subtracting the bone diameter from the total callus diameter. Each radiograph was measured three times, and the mean of the three measurements was used for statistical analysis. Radiographs were coded and evaluated by an observer blinded to treatment allocation to minimize observer bias.

Blood collection and serum preparation

Under general anesthesia, blood samples were collected directly from the heart of four animals per group on postoperative days 7 and 30. Approximately 5 mL of blood was collected from each animal into tubes without anticoagulant and transported to the laboratory. The blood samples were allowed to clot before centrifugation at 2500 rpm for 10 min. The resulting serum was transferred into separate tubes and stored at -80°C until biochemical analysis.

Assessment of oxidative stress biomarkers

Determination of CAT levels: CAT levels were determined using a commercially available enzyme-linked immunosorbent assay kit (BT Laboratory, Shanghai, China; Cat. No. E0869Ra) according to the manufacturer's instructions. All samples, standards, and reagents were brought to room temperature before analysis.

Samples and standards were added to microplate wells precoated with a rat CAT-specific monoclonal

antibody. Biotinylated detection antibody and streptavidin–horseradish peroxidase conjugate were subsequently added, and the plate was incubated at 37°C for 60 min to facilitate antigen–antibody binding. After incubation, the wells were washed five times with the supplied washing buffer to remove unbound components. Chromogenic substrate solutions A and B were added, and the plate was incubated in the dark at 37°C for 10 min. The reaction was terminated using stop solution, and absorbance was measured at 450 nm using a microplate reader within 10 min. CAT concentrations were calculated by interpolation from the standard calibration curve and expressed according to the manufacturer's instructions.

Determination of SOD levels: SOD levels were determined using a commercially available enzyme-linked immunosorbent assay kit (BT Laboratory; Cat. No. E0168Ra) according to the manufacturer's instructions. All samples, standards, and reagents were brought to room temperature before analysis.

Samples and standards were added to antibody-coated microplate wells, followed by biotinylated anti-SOD1 antibody and streptavidin–horseradish peroxidase conjugate. The plate was incubated at 37°C for 60 min to facilitate antigen–antibody binding and subsequently washed five times using the supplied washing buffer. Chromogenic substrate solutions A and B were added, followed by incubation in the dark at 37°C for 10 min. The reaction was terminated with stop solution, and absorbance was measured at 450 nm within 10 min using a microplate reader. SOD concentrations were calculated by interpolation from the standard calibration curve according to the manufacturer's instructions.

Histological examination: Femoral specimens were dissected free of surrounding soft tissues and fixed in 10% formalin for histological evaluation. Histological examinations were performed on specimens collected on postoperative days 7, 14, 21, and 30. All sections were evaluated by two pathologists blinded to treatment allocation.

Fracture healing was graded using a modified 13-point histopathological scoring system based on the proportions of fibrous tissue, cartilage (Ca), immature bone, and mature bone within the fracture site, adapted from Huo *et al.* [11] (Table 1).

Table 1: Histopathological scoring system for the evaluation of fracture healing [11].

Score	Histopathological features of callus and bone
I	Fibrous tissue or granulation tissue
II	Predominantly fibrous tissue with a small amount of cartilage (Ca)
III	Equal amounts of fibrous and Ca tissues
IV	Entirely Ca tissue
V	Predominantly Ca tissue with a small amount of immature (woven) bone
VI	Equal amounts of Ca tissue and immature bone
VII	Predominantly immature bone with a small amount of Ca
VIII	Entirely immature bone
IX	Immature bone with a small amount of mature bone
X	Mature (lamellar) bone = 5–25%; early remodeling
XI	Mature (lamellar) bone = 26–50%; mid-remodeling
XII	Mature (lamellar) bone = 51–75%; advanced-remodeling
XIII	Mature (lamellar) bone ≥75%; late remodeling
XIV	Mature (lamellar) bone = 100%; complete remodeling

Histomorphometric analysis of bone cells

Histomorphometric analysis was performed using AmScope software. Osteocytes, identified as flat or spindle-shaped cells with almond-shaped cell bodies, and osteoblasts, identified by their cuboidal or columnar morphology, were counted in 25 microscopic fields in each section from each experimental group, and mean counts were calculated. Osteoclasts were identified as large, irregular multinucleated cells containing 2–20 or more nuclei and typically exhibiting pale-purple cytoplasm with fine granules. Osteoclasts were counted within a 100-μm region in each section at 100× magnification.

Statistical analysis

Quantitative outcome variables, including postoperative callus size, CAT and SOD measurements, and histomorphometric counts of osteocytes, osteoblasts, and osteoclasts, were evaluated using parametric statistical procedures. Data distribution was assessed using the Shapiro–Wilk test, and homogeneity of variance among

groups was evaluated using Levene's test.

Two-way analysis of variance was performed with AgNP concentration (control, 2.5, 5, and 10 $\mu\text{g/mL}$) and postoperative assessment time as fixed factors. When significant main effects or concentration $\times$ time interactions were detected, Tukey's honestly significant difference test was used for post hoc multiple pairwise comparisons.

All quantitative data were expressed as mean $\pm$ standard error of the mean. For radiographic, histological, and histomorphometric assessments, three animals were evaluated per group at each postoperative time point ($n = 3$). For CAT and SOD assessments, four animals were evaluated per group on postoperative days 7 and 30 ($n = 4$). Statistical significance was established at $p < 0.05$.

RESULTS

Clinical observations

Throughout the postoperative observation period, all animals were monitored daily for general health, mobility, food and water intake, bowel movements, weight bearing ability, and surgical-site condition. No severe treatment-related clinical abnormalities; marked weight loss, persistent pain-related signs, severe infection, impaired mobility, or wound dehiscence were observed. Feeding, drinking, and bowel movements remained normal in all groups after surgery. Daily assessments also showed movement and weight bearing on the osteotomized limb in all groups.

Postmortem examination

Three rats from each group were euthanized at each experimental time point on postoperative days 7, 14, 21, and 30. After removal of the surrounding skin and soft tissues, the femora were examined macroscopically for callus formation and general progression of fracture healing. Gross observations were considered qualitative and supportive rather than primary outcome measures.

Gross findings generally corresponded with the radiographic and histological observations, with more prominent callus formation observed in the AgNP-treated groups, particularly at the highest concentration. In the control group, complete cortical continuity was not observed before day 30. In the 2.5 and 5 $\mu\text{g/mL}$ groups, apparent fracture closure was observed by day 21, followed by cortical continuity by day 30. In the 10 $\mu\text{g/mL}$ group, apparent fracture closure was observed as early as day 7, with progressive consolidation thereafter (Figure 2). These observations were interpreted qualitatively, whereas radiographic and histological assessments constituted the principal measures of fracture healing progression.

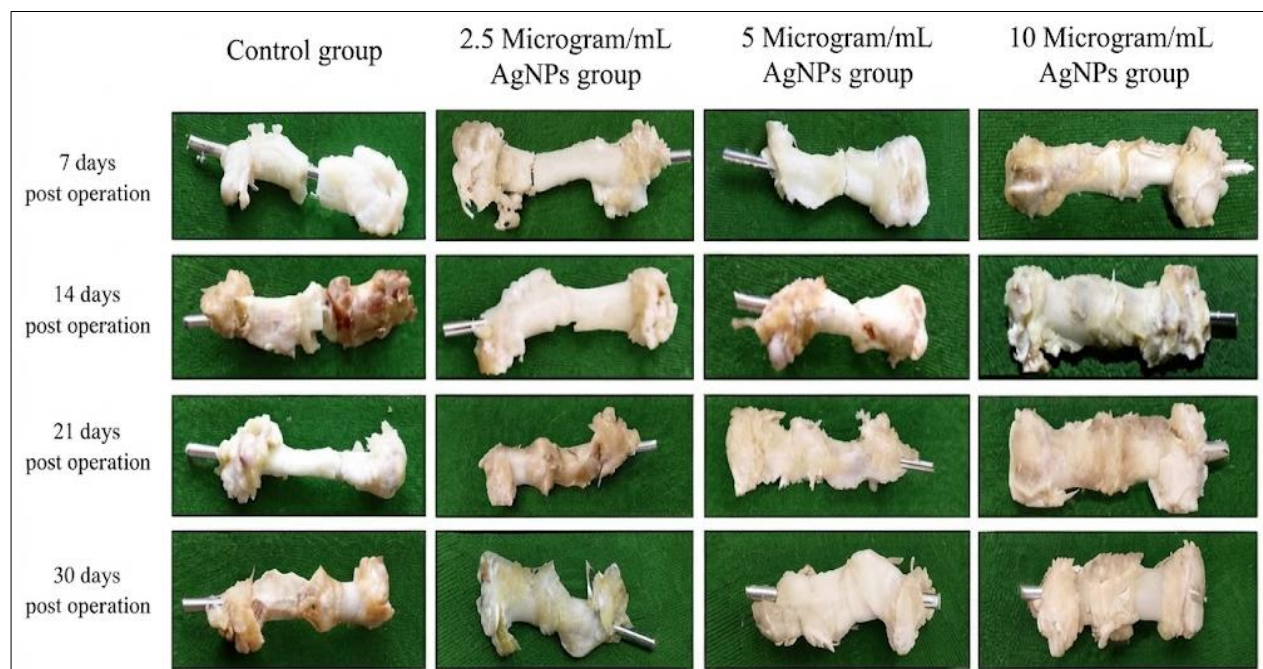


Figure 2: Representative postmortem observations of fracture healing in the control and silver nanoparticle (AgNP)-treated groups at different postoperative time points. In the control group, complete cortical continuity was not observed before day 30. In the 2.5 and 5 $\mu\text{g/mL}$ groups, apparent fracture closure was observed by day 21, with cortical continuity evident by day 30. In the 10 $\mu\text{g/mL}$ group. The 10 $\mu\text{g/mL}$ AgNPs group showed apparent early signs of fracture repair on postoperative day 7, with further progression of healing at subsequent time points.

Postoperative callus progression

Local AgNP administration was associated with concentration- and time-dependent differences in radiographic callus size during the 30-day postoperative period (Table 2). On day 7, all AgNP-treated groups had significantly greater callus sizes than the control group (1.20 ± 0.12 , 1.58 ± 0.19 , and 2.14 ± 0.16 mm in the 2.5, 5, and 10 $\mu\text{g/mL}$ groups, respectively, compared with 0.17 ± 0.01 mm in the control group; $p < 0.05$). The 10 $\mu\text{g/mL}$ group had the greatest callus size at this time point.

Callus size increased progressively with postoperative time in all groups. On day 14, the 5 and 10 $\mu\text{g/mL}$ groups were significantly larger than the control group, whereas the 2.5 $\mu\text{g/mL}$ group did not differ significantly from the control, as indicated by the superscript notation. On day 21, the 10 $\mu\text{g/mL}$ group had a significantly greater callus size than the other groups, whereas the control, 2.5 $\mu\text{g/mL}$, and 5 $\mu\text{g/mL}$ groups did not differ significantly from one another. By day 30, callus size was 5.28 ± 0.09 mm in the control group and increased sequentially to 6.20 ± 0.11 , 7.01 ± 0.15 , and 8.56 ± 0.05 mm in the 2.5, 5, and 10 $\mu\text{g/mL}$ groups, respectively, with significant differences among all four groups ($p < 0.05$).

Table 2: Effect of silver nanoparticles (AgNPs) on postoperative callus size (mm).

Treatment group	Day 7	Day 14	Day 21	Day 30
Control	$0.17 \pm 0.01^{\text{aA}}$	$3.02 \pm 0.06^{\text{aB}}$	$4.23 \pm 0.11^{\text{ac}}$	$5.28 \pm 0.09^{\text{aD}}$
2.5 $\mu\text{g/mL}$ AgNPs	$1.20 \pm 0.12^{\text{bA}}$	$3.23 \pm 0.09^{\text{aB}}$	$4.51 \pm 0.05^{\text{ac}}$	$6.20 \pm 0.11^{\text{bD}}$
5 $\mu\text{g/mL}$ AgNPs	$1.58 \pm 0.19^{\text{bA}}$	$3.50 \pm 0.15^{\text{bB}}$	$4.76 \pm 0.08^{\text{ac}}$	$7.01 \pm 0.15^{\text{cD}}$
10 $\mu\text{g/mL}$ AgNPs	$2.14 \pm 0.16^{\text{cA}}$	$4.88 \pm 0.04^{\text{cB}}$	$6.56 \pm 0.21^{\text{bc}}$	$8.56 \pm 0.05^{\text{dD}}$

Values are expressed as mean $\pm$ standard error of the mean ($n = 3/\text{group}$). Different lowercase superscripts (a–d) within the same column indicate significant differences among treatment groups ($p < 0.05$). Different uppercase superscripts (A–D) within the same row indicate significant differences among postoperative time points ($p < 0.05$).

Serial radiographic examinations supported the quantitative findings. The control group showed a progressive reduction in the fracture gap and callus formation, with faint callus evident by day 21 and partial bridging by day 30 (Figure 3). The 2.5 $\mu\text{g/mL}$ group showed progressive callus formation and reduction of the fracture gap, with partial bridging by day 21 and radiographic disappearance of the fracture gap by day 30 (Figure 4). In the 5 $\mu\text{g/mL}$ group, radiographic continuity across the fracture site was evident by day 21, followed by further cortical and medullary continuity by day 30 (Figure 5). The 10 $\mu\text{g/mL}$ group demonstrated progressive callus formation and radiographic maturation, with cortical and medullary continuity observed by day 30 (Figure 6).

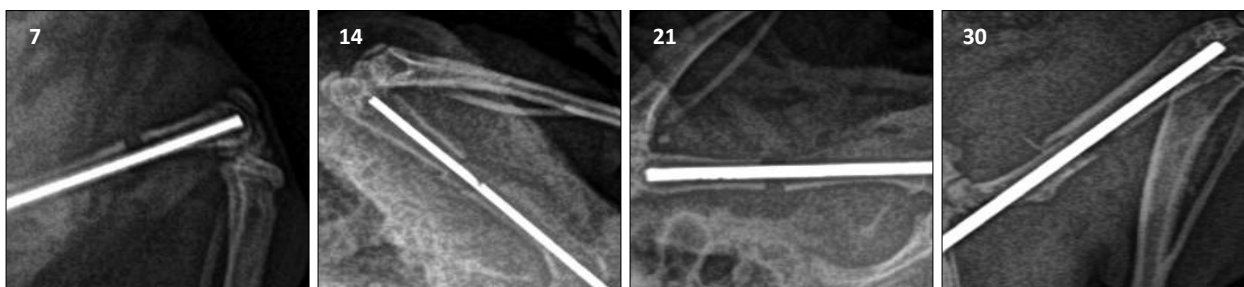


Figure 3: Representative serial radiographs of the femur in the control group on postoperative days 7, 14, 21, and 30, showing progressive reduction of the fracture gap and callus formation. Faint callus formation was observed by day 21, followed by partial bridging of the fracture site by day 30.

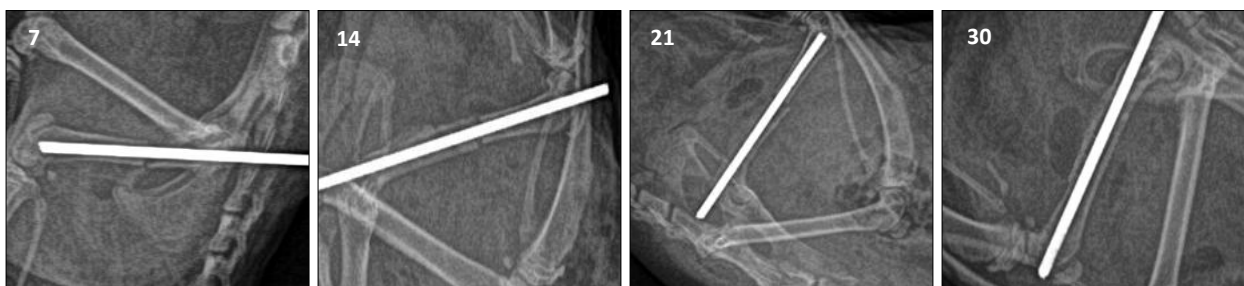


Figure 4: Representative serial radiographs of the femur in the 2.5 $\mu\text{g/mL}$ silver nanoparticles-treated group on postoperative days 7, 14, 21, and 30, showing progressive reduction of the fracture gap and increasing callus formation. Partial bridging was observed by day 21, with radiographic disappearance of the fracture gap by day 30.

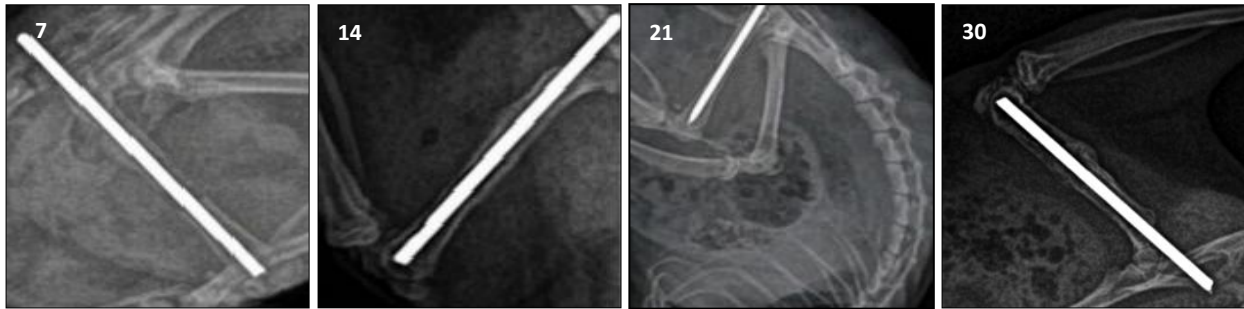


Figure 5: Representative serial radiographs of the femur in the 5 µg/mL silver nanoparticles-treated group on postoperative days 7, 14, 21, and 30. Progressive callus formation and reduction of the fracture gap were observed, with radiographic continuity across the fracture site evident by day 21 and further cortical and medullary continuity by day 30.

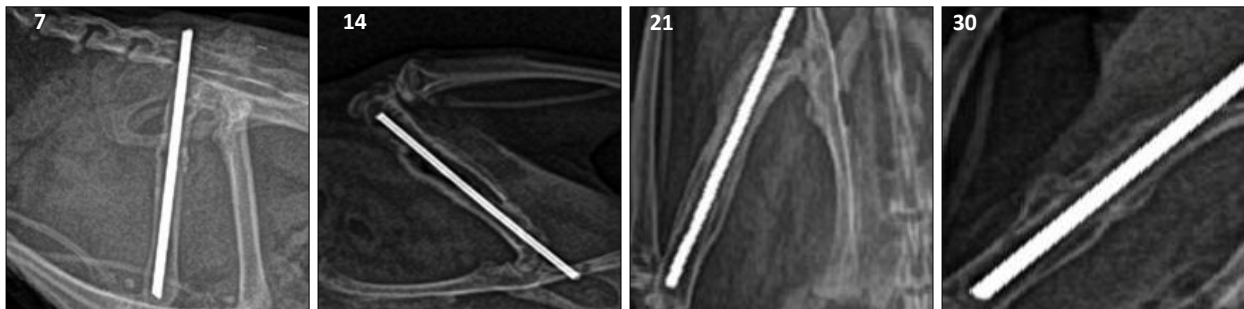


Figure 6: Representative serial radiographs of the femur in the 10 µg/mL silver nanoparticles-treated group on postoperative days 7, 14, 21, and 30, demonstrating progressive callus formation and radiographic maturation of the fracture site. Cortical and medullary continuity was observed by day 30.

Antioxidant enzyme responses

CAT and SOD showed contrasting concentration- and time-dependent patterns following AgNP administration (Table 3). On day 7, CAT increased progressively with AgNP concentration, from 20.29 ± 0.06 ng/mL in the control group to 30.39 ± 0.13 ng/mL, 36.13 ± 0.37 ng/mL, and 42.63 ± 0.22 ng/mL in the 2.5, 5, and 10 µg/mL groups, respectively ($p < 0.05$). CAT values decreased by day 30 in all groups but remained significantly higher in each AgNP-treated group than in the control group ($p < 0.05$).

In contrast, SOD values were significantly lower with increasing AgNP concentrations on day 7, declining from 6.12 ± 0.05 ng/mL in the control group to 4.60 ± 0.04 ng/mL, 4.14 ± 0.03 ng/mL, and 3.57 ± 0.01 ng/mL in the 2.5, 5, and 10 µg/mL groups, respectively ($p < 0.05$). SOD decreased further by day 30, when the corresponding values were 3.33 ± 0.02 ng/mL, 2.44 ± 0.01 ng/mL, 2.62 ± 0.01 ng/mL, and 1.43 ± 0.01 ng/mL, respectively.

Table 3: Effect of AgNPs on CAT and SOD1 levels.

Treatment group	CAT (ng/mL; day 7)	CAT (ng/mL; day 30)	SOD (ng/mL; day 7)	SOD (ng/mL; day 30)
Control	20.29 ± 0.06^{aA}	15.31 ± 0.14^{aB}	6.12 ± 0.05^{aA}	3.33 ± 0.02^{aB}
2.5 µg/mL AgNPs	30.39 ± 0.13^{bA}	24.47 ± 0.20^{bB}	4.60 ± 0.04^{bA}	2.44 ± 0.01^{bB}
5 µg/mL AgNPs	36.13 ± 0.37^{cA}	26.49 ± 0.09^{cB}	4.14 ± 0.03^{cA}	2.62 ± 0.01^{cB}
10 µg/mL AgNPs	42.63 ± 0.22^{dA}	28.35 ± 0.19^{dB}	3.57 ± 0.01^{dA}	1.43 ± 0.01^{dB}

Values are expressed as mean $\pm$ standard error of the mean ($n = 4/\text{group}$). Different lowercase superscripts (a–d) within the same column indicate significant differences among treatment concentrations ($p < 0.05$). Different uppercase superscripts (A–B) indicate significant differences between days 7 and 30 for the same enzyme ($p < 0.05$). AgNPs = Silver nanoparticles; CAT = Catalase; SOD = Superoxide dismutase.

Histomorphometric bone-cell dynamics

Histomorphometric analysis demonstrated temporal and concentration-dependent differences in osteoclast, osteoblast, and osteocyte counts. Osteoclasts were not detected in any group on day 7. By day 14, osteoclast counts were highest in the 10 µg/mL group (10.00 ± 1.15), followed by the 5 µg/mL group (6.00 ± 1.15), indicating an earlier osteoclast response at the higher AgNP concentrations. These counts subsequently declined by day 21 to 1.67 ± 0.33 and 2.33 ± 0.88 in the 10 and 5 µg/mL groups, respectively. In contrast, osteoclast counts peaked later, on day 21, in the 2.5 µg/mL and control groups, reaching 6.67 ± 0.67 and 3.00 ± 0.58 , respectively (Figure 7).

Osteoblast counts increased during the later stages of fracture healing. On day 30, values were 5.53 ± 0.69 ,

7.87 ± 0.58 , 10.73 ± 0.80 , and 10.80 ± 0.84 in the control, 2.5, 5, and 10 $\mu\text{g}/\text{mL}$ groups, respectively (Figure 8). Osteocyte counts showed a similar concentration-related pattern on day 30, reaching 3.60 ± 0.66 , 8.53 ± 0.69 , 10.14 ± 0.66 , and 12.03 ± 1.39 in the respective groups (Figure 9).

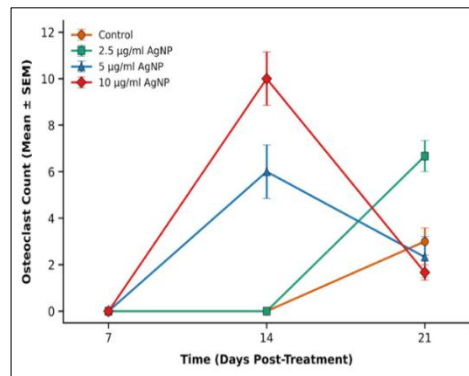


Figure 7: Temporal variation in osteoclast counts following local silver nanoparticle administration. Osteoclast counts were assessed on postoperative days 7, 14, and 21 in the control, 2.5, 5, and 10 $\mu\text{g}/\text{mL}$ groups. Data are presented as mean $\pm$ standard error of the mean ($n = 3$ animals/group/time point). The 5 and 10 $\mu\text{g}/\text{mL}$ groups showed an earlier increase in osteoclast counts, peaking on day 14, whereas the control and 2.5 $\mu\text{g}/\text{mL}$ groups showed later peaks on day 21. Statistical analysis was performed using two-way analysis of variance followed by Tukey's honestly significant difference post hoc test. A p -value < 0.05 was considered statistically significant. Significant comparisons included day 14 (10 $\mu\text{g}/\text{mL}$ vs. control, $p < 0.001$; 10 $\mu\text{g}/\text{mL}$ vs. 5 $\mu\text{g}/\text{mL}$, $p = 0.018$) and day 21 (2.5 $\mu\text{g}/\text{mL}$ vs. control, $p = 0.024$).

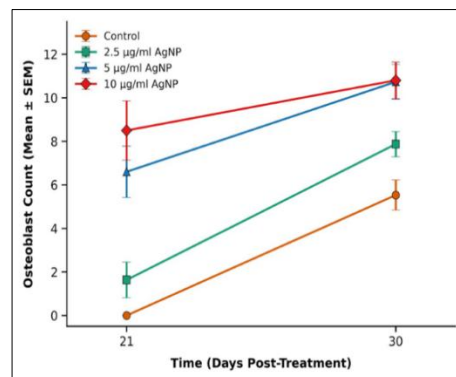


Figure 8: Osteoblast counts during late-stage fracture healing following local silver nanoparticle administration. Osteoblast counts were assessed on postoperative days 21 and 30 in the control, 2.5, 5, and 10 $\mu\text{g}/\text{mL}$ groups. Data are presented as mean $\pm$ standard error of the mean ($n = 3$ animals/group/time point). Statistical analysis was performed using two-way analysis of variance followed by Tukey's honestly significant difference post hoc test. A p -value < 0.05 was considered statistically significant. Significant differences were observed on day 30 (10 $\mu\text{g}/\text{mL}$ vs. control, $p < 0.001$; 5 $\mu\text{g}/\text{mL}$ vs. control, $p < 0.001$).

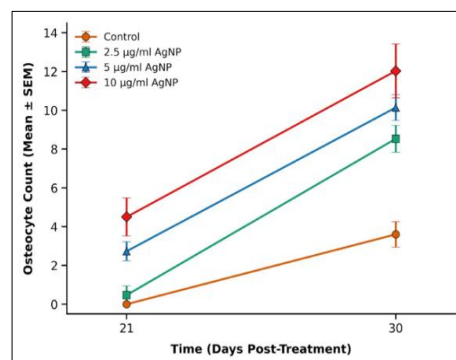


Figure 9: Osteocyte counts during tissue maturation following local silver nanoparticle administration. Osteocyte counts were assessed on postoperative days 21 and 30 in the control, 2.5, 5, and 10 $\mu\text{g}/\text{mL}$ groups. Data are presented as mean $\pm$ standard error of the mean ($n = 3$ animals/group/time point). Statistical analysis was performed using two-way analysis of variance followed by Tukey's honestly significant difference post hoc test. A p -value < 0.05 was considered statistically significant. On day 30, significant differences were observed between the 10 $\mu\text{g}/\text{mL}$ and control groups ($p < 0.001$), the 5 $\mu\text{g}/\text{mL}$ and control groups ($p < 0.001$), and the 2.5 $\mu\text{g}/\text{mL}$ and control groups ($p = 0.003$).

Histological evaluation of femoral fracture healing

Postoperative day 7: On day 7, the control group showed predominantly hematoma and fibrous granulation tissue at the fracture site, corresponding to score I (Figure 10). The 2.5 $\mu\text{g}/\text{mL}$ group showed fibrous granulation tissue with a small amount of Ca, corresponding to score II (Figure 11). The 5 $\mu\text{g}/\text{mL}$ group showed approximately equal proportions of fibrous and Ca tissues, corresponding to score III (Figure 12). The 10 $\mu\text{g}/\text{mL}$ group demonstrated approximately equal amounts of Ca and immature bone, corresponding to score VI (Figure 13).

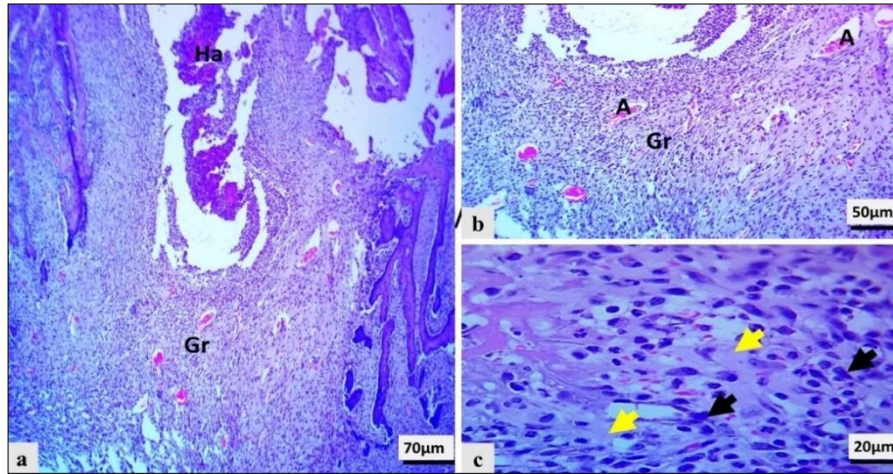


Figure 10: Light microscopic sections of the femoral fracture site in the control group on postoperative day 7 showing score I features. (a) The fracture site is covered by hematoma (Ha), with incomplete union and a gap containing fibrous granulation tissue. (b and c) Fibrous granulation tissue (Gr) containing numerous inflammatory cells (black arrows), newly formed blood vessels (A), and collagen deposition (yellow arrows). Hematoxylin and eosin staining.

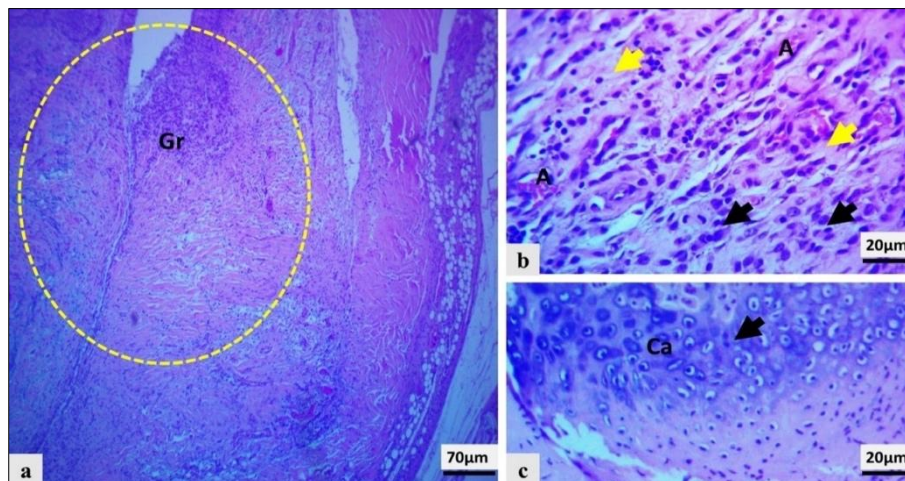


Figure 11: Light microscopic sections of the femoral fracture site in the 2.5 $\mu\text{g}/\text{mL}$ silver nanoparticles-treated group on postoperative day 7 showing score II features. (a–c) Partial fracture union with fibrous granulation tissue and a small amount of cartilage. (b) Fibrous granulation tissue (Gr) containing numerous inflammatory cells (black arrows), newly formed blood vessels, and collagen deposition (yellow arrows). (c) A small amount of cartilage callus (Ca) within the fracture gap. Hematoxylin and eosin staining.

Postoperative day 14: On day 14, the control group showed predominantly fibrous granulation tissue with a small amount of Ca, corresponding to score II (Figure 14). The 2.5 $\mu\text{g}/\text{mL}$ group showed predominantly Ca callus with hypertrophied chondrocytes and limited residual fibrous granulation tissue, corresponding to score IV (Figure 15). The 5 $\mu\text{g}/\text{mL}$ group demonstrated Ca with a small amount of immature bone and features of chondro-ossification, corresponding to score V (Figure 16). The 10 $\mu\text{g}/\text{mL}$ group showed predominantly immature trabecular bone (TB) containing osteocytes and osteoblasts, corresponding to score VIII (Figure 17).

Postoperative day 21: On day 21, the control group showed predominantly immature bone with a small amount of Ca, corresponding to score VII (Figure 18). The 2.5 $\mu\text{g}/\text{mL}$ group showed predominantly immature TB, corresponding to score VIII (Figure 19). Both the 5 and 10 $\mu\text{g}/\text{mL}$ groups showed features of early remodeling, with immature and mature bone, developing bone marrow (BM), and cortical organization, corresponding to score IX (Figures 20 and 21).

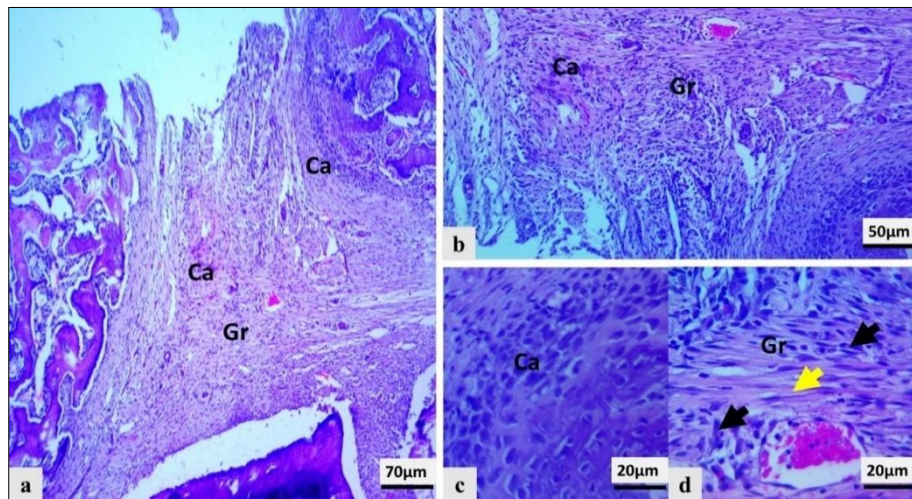


Figure 12: Light microscopic sections of the femoral fracture site in the 5 µg/mL silver nanoparticles-treated group on postoperative day 7 showing score III features. (a and b) The fracture site contains approximately equal amounts of fibrous granulation tissue and cartilage. (c) Cartilage callus (Ca) arising from the periosteal region. (d) Fibrous granulation tissue (Gr) containing numerous inflammatory cells (black arrows), newly formed blood vessels, and collagen deposition (yellow arrows). Hematoxylin and eosin staining.

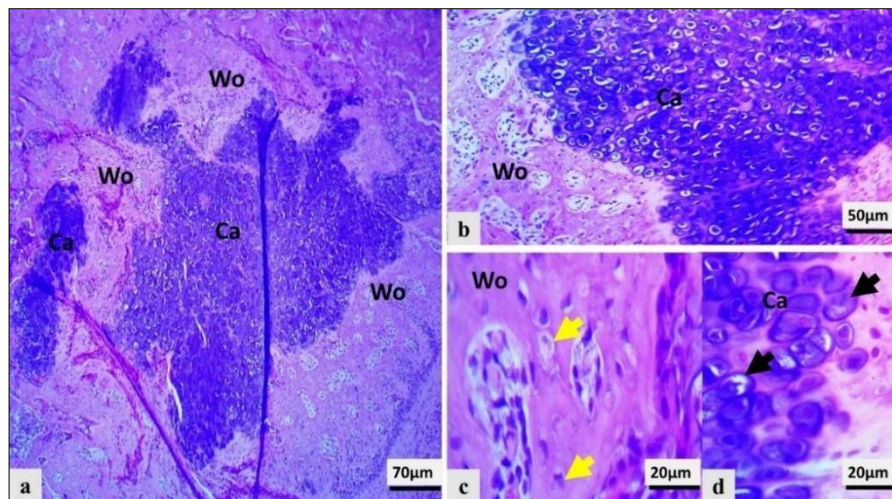


Figure 13: Light microscopic sections of the femoral fracture site in the 10 µg/mL silver nanoparticles-treated group on postoperative day 7 showing score VI features. (a and b) Fracture union characterized by approximately equal amounts of cartilage (Ca) and immature woven bone (Wo). (c) Wo formed through endochondral ossification, containing immature osteocytes (yellow arrows). (d) Hypertrophied chondrocytes (black arrows). Hematoxylin and eosin staining.

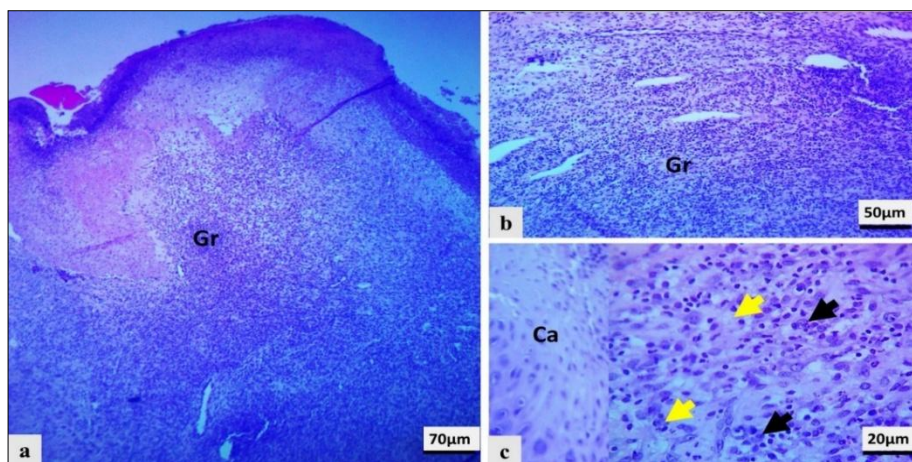


Figure 14: Light microscopic sections of the femoral fracture site in the control group on postoperative day 14 showing score II features. (a–c) Fracture union with fibrous granulation tissue and a small amount of cartilage (Ca). (c) Fibrous granulation tissue (Gr) containing numerous inflammatory cells (black arrows), newly formed blood vessels, and collagen deposition (yellow arrows). Hematoxylin and eosin staining.

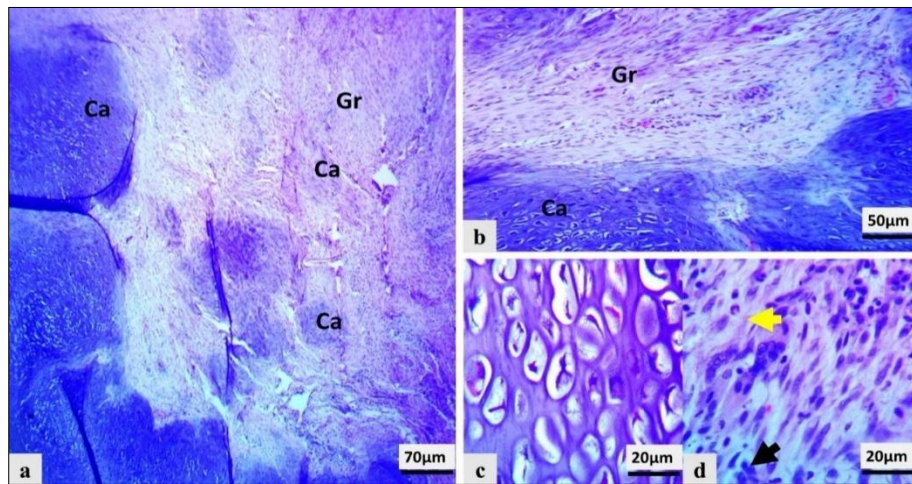


Figure 15: Light microscopic sections of the femoral fracture site in the 2.5 µg/mL silver nanoparticles-treated group on postoperative day 14 showing score IV features. (a and b) The fracture site is predominantly occupied by cartilage (Ca), with limited fibrous granulation tissue. (c) Hypertrophied chondrocytes. (d) Fibrous granulation tissue (Gr) containing inflammatory cells (black arrows) and collagen deposition (yellow arrows). Hematoxylin and eosin staining.

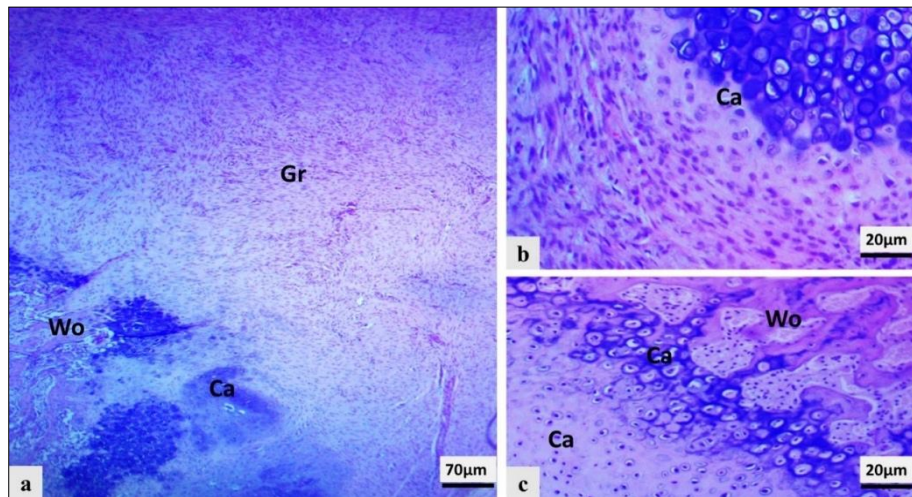


Figure 16: Light microscopic sections of the femoral fracture site in the 5 µg/mL silver nanoparticles-treated group on postoperative day 14 showing score V features. (a and b) The healed fracture region contains fibrous granulation tissue (Gr), cartilage (Ca), and a small amount of immature woven bone (Wo). (c) Features of chondro-ossification. Hematoxylin and eosin staining.

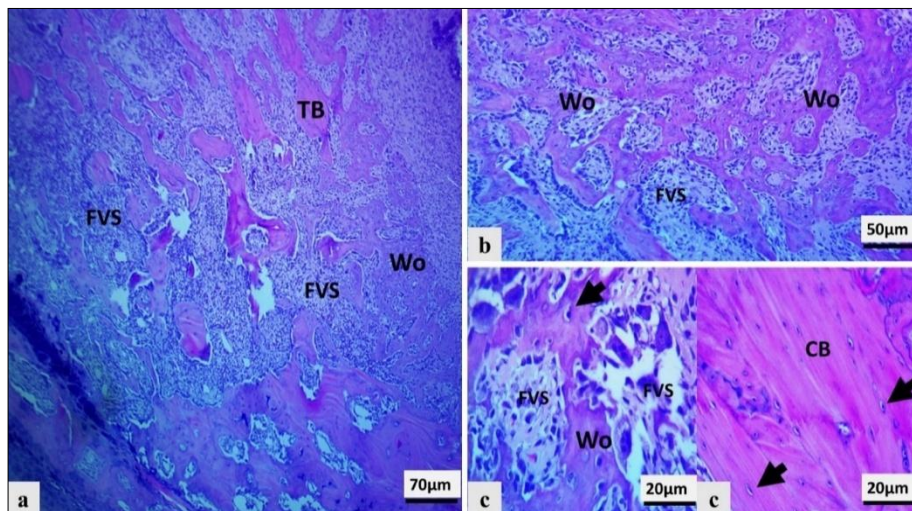


Figure 17: Light microscopic sections of the femoral fracture site in the 10 µg/mL silver nanoparticles-treated group on postoperative day 14 showing score VIII features. (a and b) The healed fracture region contains immature trabecular bone (TB), osteocytes (black arrows), osteoblasts, and intervening fibrous vascular stroma (FVS). (c) Immature cortical bone (CB) without fully formed Haversian canals. Hematoxylin and eosin staining.

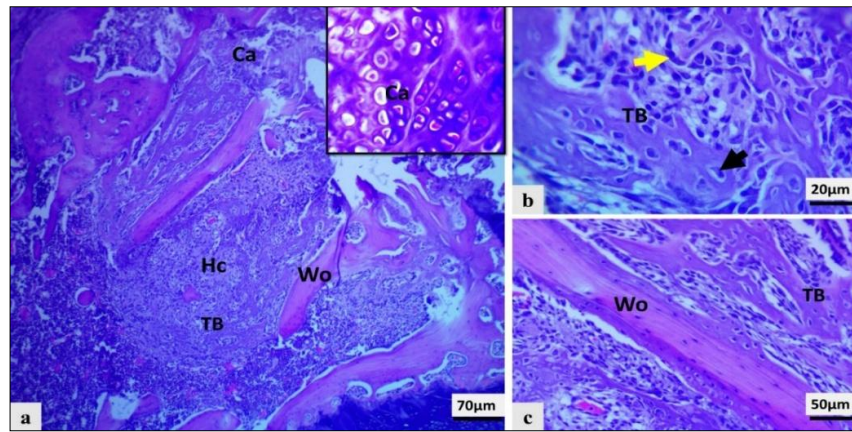


Figure 18: Light microscopic sections of the femoral fracture site in the control group on postoperative day 21 showing score VII features. (a–c) The healed fracture region contains predominantly immature bone trabeculae (Wo) with a small amount of cartilage callus (Ca and inset). (b and c) Osteocytes (black arrows) are present within the trabeculae, and osteoblasts (yellow arrows) line the endosteal surface. Hematoxylin and eosin staining.

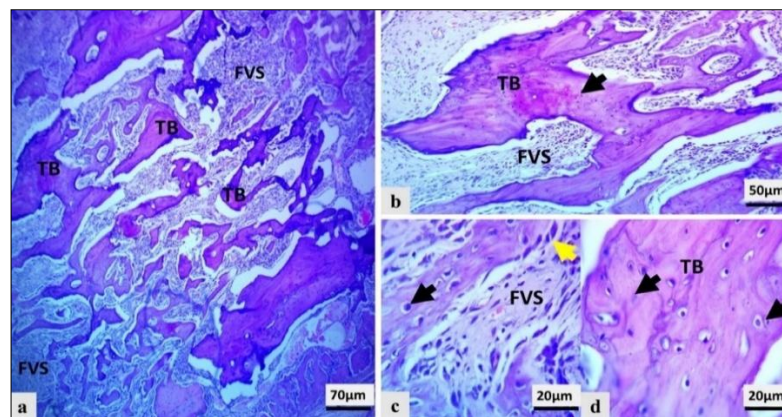


Figure 19: Light microscopic sections of the femoral fracture site in the 2.5 µg/mL silver nanoparticles-treated group on postoperative day 21 showing score VIII features. (a and b) The healed fracture region contains predominantly immature trabecular bone (TB) separated by fibrous vascular stroma (FVS). (b–d) Immature TB shows a relatively unorganized mineralized matrix, immature osteocytes (black arrows), and osteoblasts (yellow arrows). Hematoxylin and eosin staining.

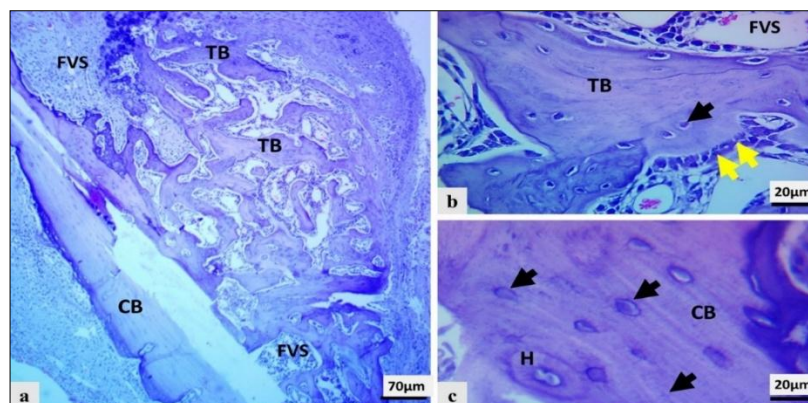


Figure 20: Light microscopic sections of the femoral fracture site in the 5 µg/mL silver nanoparticles-treated group on postoperative day 21 showing score IX features of early remodeling. (a–c) The healed fracture region contains predominantly immature trabecular bone (TB) separated by bone marrow (BM), together with a small amount of mature bone. (b) Mature TB shows an organized mineralized matrix, mature osteocytes (black arrows), and osteoblasts (yellow arrows). (c) Immature cortical bone contains a developing Haversian canal (H) and osteocytes (black arrow). Hematoxylin and eosin staining.

Postoperative day 30: On day 30, the control group showed early remodeling characterized predominantly by immature TB and fibrous vascular stroma, corresponding to score IX (Figure 22). The 2.5 and 5 µg/mL groups showed mid-remodeling, with approximately 50% mature TB, BM development, and partially matured cortical bone, corresponding to score XI (Figures 23 and 24). The 10 µg/mL group showed more advanced remodeling, with approximately 75% mature TB and organized cortical bone, corresponding to score XII (Figure 25).

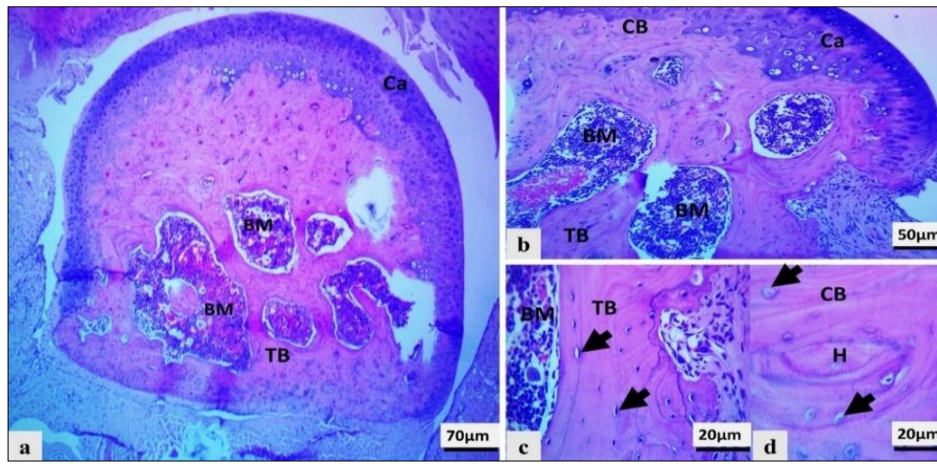


Figure 21: Light microscopic sections of the femoral fracture site in the 10 µg/mL silver nanoparticles-treated group on postoperative day 21 showing score IX features of early remodeling. (a) The healed fracture region contains trabecular bone (TB), bone marrow (BM), and a small amount of mature bone surrounded by hyaline cartilage (Ca). (c) Immature TB contains a less-organized mineralized matrix and osteocytes (black arrows). (d) Immature cortical bone contains a Haversian canal (H) surrounded by concentric lamellae containing osteocytes (black arrow). Hematoxylin and eosin staining.

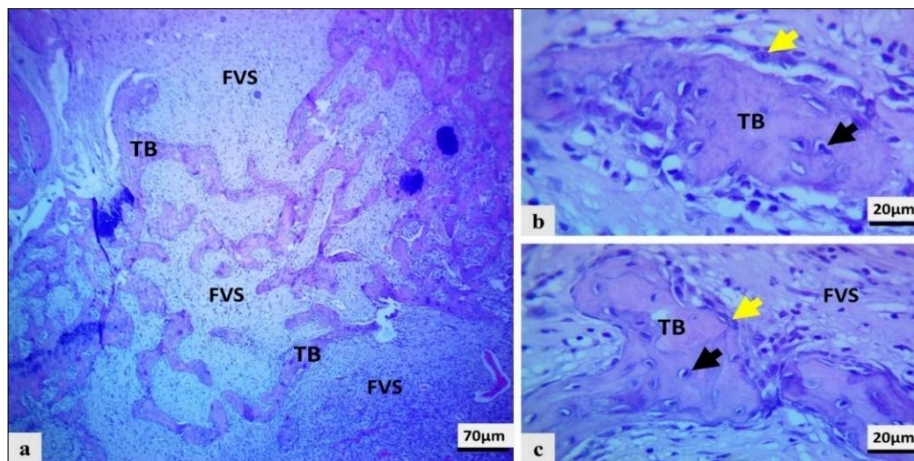


Figure 22: Light microscopic sections of the femoral fracture site in the control group on postoperative day 30 showing score IX features of early remodeling. (a–c) The healed fracture region contains predominantly immature trabecular bone (TB) separated by fibrous vascular stroma (FVS). (b and c) Immature TB contains a less-organized mineralized matrix, osteocytes (black arrows), and osteoblasts (yellow arrows). Hematoxylin and eosin staining.

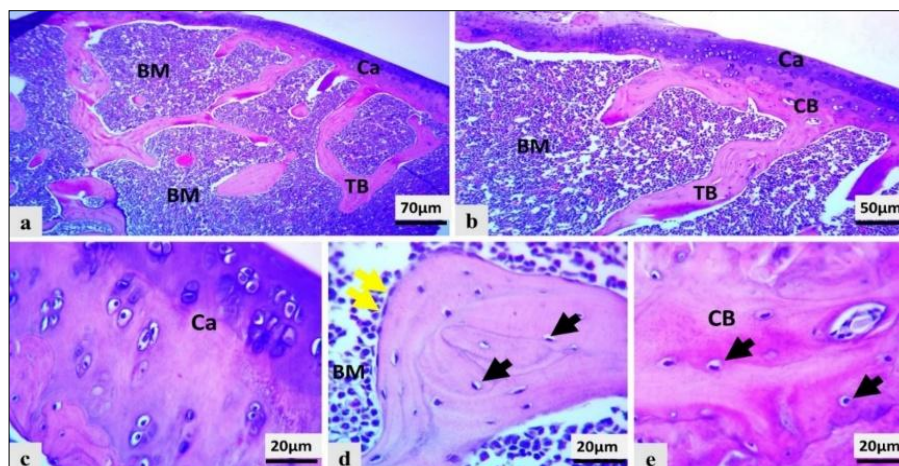


Figure 23: Light microscopic sections of the femoral fracture site in the 2.5 µg/mL silver nanoparticles-treated group on postoperative day 30 showing score XI features of mid-remodeling. (a and b) The healed fracture region contains approximately 50% organized mature trabecular bone (TB), separated by bone marrow (BM) and surrounded by hyaline cartilage (Ca). (c) Mature TB contains an organized mineralized matrix, osteocytes (black arrows), and osteoblasts (yellow arrows). (d) Early mature cortical bone contains a Haversian canal (H) surrounded by concentric lamellae containing osteocytes (black arrow). Hematoxylin and eosin staining.

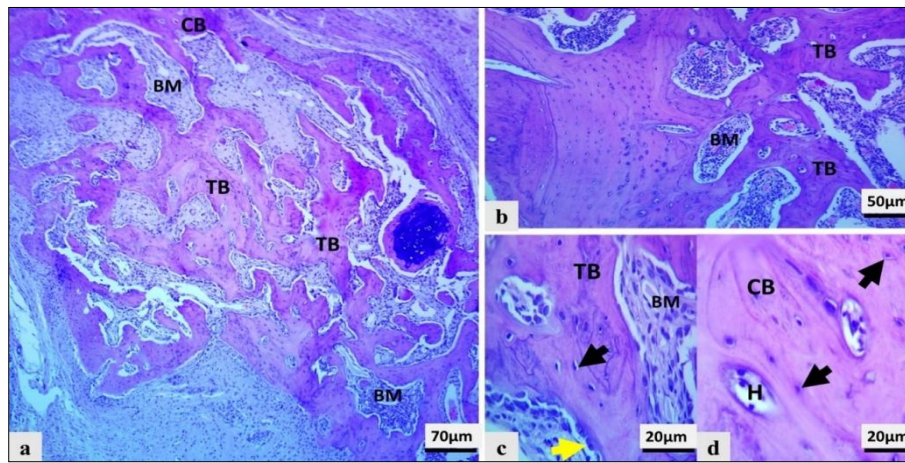


Figure 24: Light microscopic sections of the femoral fracture site in the 5 µg/mL silver nanoparticles-treated group on postoperative day 30 showing score XI features of mid-remodeling. (a and b) The healed fracture region contains approximately 50% organized mature trabecular bone (TB) separated by bone marrow (BM). (c) Mature TB contains an organized mineralized matrix, osteocytes (black arrows), and osteoblasts (yellow arrow). (d) Partially matured cortical bone contains a Haversian canal (H) surrounded by concentric lamellae containing osteocytes (black arrow). Hematoxylin and eosin staining.

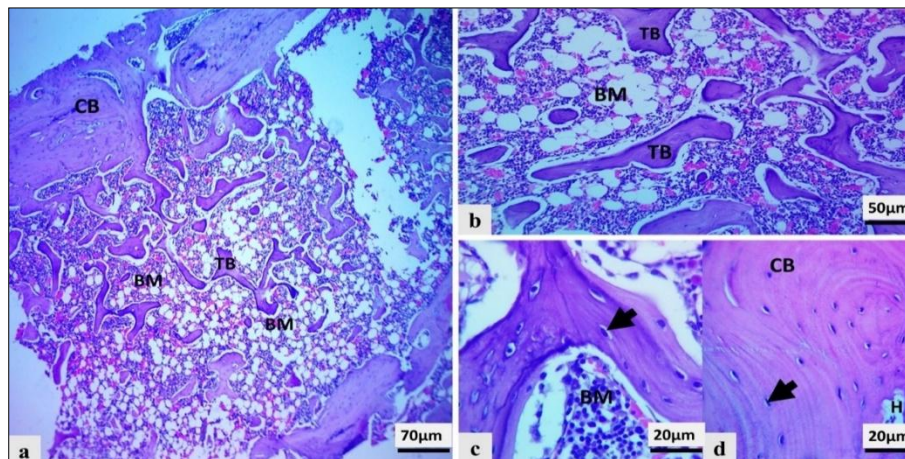


Figure 25: Light microscopic sections of the femoral fracture site in the 10 µg/mL silver nanoparticles-treated group on postoperative day 30 showing score XII features of advanced remodeling. (a and b) The healed fracture region contains approximately 75% organized mature trabecular bone (TB) separated by bone marrow (BM). (c) Mature TB contains a well-organized mineralized matrix and osteocytes (black arrows). (d) Mature cortical bone contains a Haversian canal (H) surrounded by multiple concentric lamellae containing osteocytes (black arrow). Hematoxylin and eosin staining.

DISCUSSION

Effects of local AgNP administration on radiographic fracture healing

Fracture healing is a highly synchronized and temporally regulated process involving an initial inflammatory response, recruitment and differentiation of mesenchymal progenitor cells, fracture callus formation and mineralization, and subsequent remodeling through coordinated osteoblast and osteoclast activity [12, 13]. The principal finding of the present study was that a single local administration of AgNPs at concentrations of 2.5, 5, and 10 µg/mL was associated with more rapid radiographic and histomorphometric progression of fracture healing in a stabilized rat femoral osteotomy model. The response generally varied with concentration and postoperative time, with the 10 µg/mL group showing the most pronounced radiographic callus formation and more advanced histological changes.

On day 7, callus size was 0.17 ± 0.01 mm in the control group compared with 1.20 ± 0.12 , 1.58 ± 0.19 , and 2.14 ± 0.16 mm in the 2.5, 5, and 10 µg/mL groups, respectively ($p < 0.05$). These differences became more pronounced during follow-up. By day 30, callus size reached 5.28 ± 0.09 , 6.20 ± 0.11 , 7.01 ± 0.15 , and 8.56 ± 0.05 mm in the control, 2.5, 5, and 10 µg/mL groups, respectively ($p < 0.05$). The progressive increase in callus size is consistent with the expected transition from early callus formation toward mineralized hard callus and subsequent remodeling [12, 14]. Importantly, these findings show an association between local AgNP exposure

and more rapid radiographic progression, but they do not establish that AgNPs directly induced osteogenesis.

Serial radiographic observations further supported differences in the temporal progression of fracture repair. The control group showed faint callus formation by day 21 and partial bridging by day 30. The 2.5 µg/mL group showed partial bridging by day 21 and radiographic disappearance of the fracture gap by day 30. In the 5 µg/mL group, radiographic continuity across the fracture site was evident by day 21, followed by further cortical and medullary continuity by day 30. The 10 µg/mL group demonstrated progressive callus maturation, with cortical and medullary continuity observed by day 30.

These observations are broadly consistent with previous experimental evidence involving AgNP-containing materials. Zhang *et al.* [1] showed in a mouse femoral fracture model that collagen-encapsulated AgNPs promoted fracture callus formation and facilitated early fracture gap closure, with evidence suggesting mesenchymal stem-cell recruitment, proliferation, and osteogenic differentiation. Han *et al.* [15] reported that an AgNP/gelatin hydrogel formulation enhanced osteoblast survival and spreading and influenced fracture healing. Similarly, Sabry *et al.* [2] reported osteogenic potential of AgNPs in critical-sized mandibular bone defects in rats. Nevertheless, direct comparison requires caution because these studies differed in nanoparticle formulation, concentration, delivery system, and experimental model [1, 2, 15].

Histomorphometric changes during fracture healing

Histomorphometric findings provided additional evidence of temporal differences in bone-cell populations following AgNP administration. Osteoclasts were not detected in any group on day 7. By day 14, osteoclast counts reached 10.00 ± 1.15 cells/high-power field (HPF) in the 10 µg/mL group and 6.00 ± 1.15 cells/HPF in the 5 µg/mL group, followed by lower values on day 21. In contrast, osteoclast counts in the 2.5 µg/mL and control groups reached their highest observed values on day 21, at 6.67 ± 0.67 and 3.00 ± 0.58 cells/HPF, respectively. Osteoclast-mediated resorption is coordinated with osteoblast-mediated bone formation during normal remodeling [13, 14]. Therefore, the earlier increase in osteoclast counts at the higher AgNP concentrations may be consistent with an earlier transition toward remodeling. However, osteoclast numbers alone do not quantify functional bone resorption, so this interpretation should be treated cautiously.

Differences in osteoblast and osteocyte populations were particularly evident on day 30. Osteoblast counts were 5.53 ± 0.69 , 7.87 ± 0.58 , 10.73 ± 0.80 , and 10.80 ± 0.84 cells/HPF in the control, 2.5, 5, and 10 µg/mL groups, respectively. Similarly, osteocyte counts increased across these groups, reaching 3.60 ± 0.66 , 8.53 ± 0.69 , 10.14 ± 0.66 , and 12.03 ± 1.39 cells/HPF, respectively. Osteoblasts produce and mineralize bone matrix, whereas osteocytes are mature bone cells embedded within the mineralized matrix and participate in bone homeostasis and remodeling [14, 16]. Thus, the higher osteoblast and osteocyte counts observed at the higher AgNP concentrations are consistent with more advanced cellular and tissue maturation at the evaluated time point.

These findings are also compatible with previous experimental observations of AgNP-associated osteogenic responses. Zhang *et al.* [1] reported enhanced fracture healing, along with increased mesenchymal stem-cell proliferation and osteogenic differentiation, in mice exposed to AgNPs. He *et al.* [17] further reported that AgNPs promoted osteogenic differentiation of human mesenchymal stem cells through autophagy-associated mechanisms. Nevertheless, the present study did not directly evaluate progenitor-cell proliferation, differentiation pathways, or molecular markers of osteogenesis; therefore, the mechanisms described in previous studies cannot be assumed to account for the present findings.

Relevance of AgNP physicochemical characteristics

The physicochemical characteristics of AgNPs are important when comparing the present findings with those of previous studies. Transmission electron microscopy showed that the AgNPs used in this study were predominantly spherical, with a primary particle size of approximately 20 nm, whereas dynamic light scattering showed a Z-average hydrodynamic diameter of 33.13 nm and a polydispersity index of 0.419. These measurements are not inherently contradictory because dynamic light scattering measures the hydrodynamic behavior of particles or particle assemblies dispersed in liquid and may be influenced by hydration, aggregation, and dispersion characteristics [18, 19].

The primary particle size in the present study is particularly relevant to previous investigations using AgNPs of a similar size. Qin *et al.* [20] evaluated AgNPs with a mean particle size of approximately 19.4 ± 5.2 nm and reported that an appropriate concentration promoted osteogenic differentiation of human urine-derived stem cells. Samberg *et al.* [21] investigated cellular uptake of 10- and 20-nm AgNPs by human adipose-derived stem cells and found that effects on cellular viability and differentiation depended on concentration and experimental

conditions. These studies provide useful context because the approximately 20-nm primary particle size used here falls within a comparable range. However, similar primary particle size does not establish equivalence among AgNP formulations or predict identical biological effects.

PVP was used as the stabilizing agent in the present study. Surface stabilization and coating can influence nanoparticle dispersion, stability, cellular interactions, uptake, and toxicity [19, 22]. Previous investigations involving AgNPs of approximately 30 nm have reported both osteogenic and cytotoxic effects under different experimental conditions [17, 19]. Consequently, the approximately 20-nm primary particle size determined by transmission electron microscopy should be distinguished from the 33.13-nm hydrodynamic diameter measured by dynamic light scattering and from nominal particle sizes reported for other AgNP formulations. Therefore, the present findings should not be generalized to all AgNP formulations solely on the basis of particle size.

Potential mechanisms associated with AgNP exposure

Several mechanisms described in previous research could contribute to the observed fracture-healing pattern. One is the established antimicrobial activity of AgNPs. AgNPs may exert antimicrobial effects by interacting with microbial membranes, disrupting cellular enzymes and respiratory processes, generating reactive oxygen species, and releasing Ag⁺ ions [4, 5, 23]. Because microbial contamination and infection can interfere with fracture healing [24, 25], antimicrobial activity could theoretically contribute to a local environment favorable to tissue repair. However, the present study did not measure bacterial burden or infection-related inflammatory responses; therefore, these findings cannot demonstrate an antimicrobial mechanism.

A second potential mechanism involves effects on cells participating in bone regeneration. Previous studies show that AgNPs can influence mesenchymal stem-cell proliferation and osteogenic differentiation, with some models implicating transforming growth factor-beta/bone morphogenetic protein-related signaling [1, 17]. Modulation of inflammatory and vascular responses represents another possible mechanism. A controlled inflammatory response is necessary during early fracture repair, whereas excessive or prolonged inflammation may interfere with subsequent regeneration [24, 26]. Angiogenesis is similarly important for supplying oxygen, nutrients, progenitor cells, and other factors required for bone formation [27, 28]. However, inflammatory cytokines, vascular density, macrophage polarization, vascular endothelial growth factor, cluster of differentiation 31, bone morphogenetic protein 2, runt-related transcription factor 2, alkaline phosphatase, and osteocalcin were not evaluated. Therefore, effects on inflammation, angiogenesis, or specific osteogenic signaling pathways remain potential mechanisms suggested by previous literature rather than mechanisms demonstrated by the present study [2, 29, 30].

Antioxidant enzyme responses

AgNP administration was associated with concentration-dependent changes in CAT levels. CAT levels increased with AgNP concentration. On day 7, CAT values increased from 20.29 ± 0.06 ng/mL in the control group to 30.39 ± 0.13 ng/mL, 36.13 ± 0.37 ng/mL, and 42.63 ± 0.22 ng/mL in the 2.5, 5, and 10 µg/mL groups, respectively ($p < 0.05$). Although CAT levels decreased in all groups by day 30, values remained higher in the AgNP-treated groups, reaching 28.35 ± 0.19 ng/mL in the 10 µg/mL group compared with 15.31 ± 0.14 ng/mL in the control group.

In contrast, SOD levels showed an inverse concentration-related pattern. On day 7, SOD values decreased from 6.12 ± 0.05 in the control group to 4.60 ± 0.04 ng/mL, 4.14 ± 0.03 ng/mL, and 3.57 ± 0.01 ng/mL in the 2.5, 5, and 10 µg/mL groups, respectively. By day 30, the corresponding values were 3.33 ± 0.02 ng/mL, 2.44 ± 0.01 ng/mL, 2.62 ± 0.01 ng/mL, and 1.43 ± 0.01 ng/mL.

The divergent CAT and SOD responses should not be interpreted simply as enhanced antioxidant defense or reduced oxidative stress. SOD participates in the conversion of superoxide radicals to hydrogen peroxide, whereas CAT contributes to the subsequent conversion of hydrogen peroxide to water and oxygen [31, 32]. Therefore, the concurrent increase in CAT and decrease in SOD indicates an altered antioxidant enzyme profile rather than a uniform improvement in antioxidant capacity. Previous studies have shown that AgNP exposure can modify reactive oxygen species production and antioxidant responses, with the magnitude and direction of these effects depending on particle characteristics, concentration, and exposure conditions [33, 34]. Because reactive oxygen species, lipid peroxidation, malondialdehyde, glutathione, and other direct indicators of oxidative damage were not measured, the present CAT and SOD findings should be interpreted as changes in antioxidant enzyme measurements rather than evidence of either oxidative injury or protection.

Concentration-dependent response and safety considerations

The findings at 10 µg/mL warrant particular attention. On day 30, this group had the largest callus size (8.56 ± 0.05 mm), highest osteoblast count (10.80 ± 0.84 cells/HPF), and highest osteocyte count (12.03 ± 1.39 cells/HPF). At the same time, it showed the highest CAT value and lowest SOD value among the experimental groups. Thus, the concentration associated with the most pronounced radiographic and histomorphometric findings also showed the greatest alteration in the measured antioxidant enzyme profile.

This concurrence does not establish that changes in CAT or SOD caused the fracture healing response. Cellular viability, apoptosis, reactive oxygen species, and tissue oxidative damage were not directly measured. Previous studies have demonstrated that AgNPs can exert concentration- and exposure-dependent cellular effects, including reactive oxygen species generation, impaired cellular viability, mitochondrial dysfunction, apoptosis, and necrosis under certain experimental conditions [19, 22]. However, differences in AgNP formulation, concentration, exposure conditions, and experimental models prevent direct extrapolation of those findings to the present study. Accordingly, although 10 µg/mL showed the most pronounced healing-related findings among the concentrations evaluated, the present data cannot establish whether this represents an optimal therapeutic concentration or is associated with subclinical cellular stress. Future dose-ranging studies should incorporate direct measurements of cellular viability, apoptosis, oxidative damage, Ag⁺ release, and tissue silver accumulation to define the therapeutic window and long-term safety of local AgNP administration.

The perioperative environment is also relevant because surgical stress, anesthesia, postoperative inflammation, and analgesic treatment can influence both fracture repair and oxidative stress-related outcomes. Controlled inflammation is integral to fracture healing, and pharmacological modulation of inflammatory responses can alter bone repair [25, 26]. Kılıç and Kozacı [8] reported that postoperative administration of different nonsteroidal anti-inflammatory drugs in rats could alter oxidative stress-related parameters, illustrating how postoperative pharmacological treatment can confound antioxidant outcomes. Therefore, standardizing anesthesia, perioperative monitoring, postoperative care, and analgesia is important when evaluating oxidative and fracture-healing responses [7, 8].

The present study used ketamine–xylazine anesthesia, perioperative monitoring, and daily postoperative clinical assessment. However, postoperative analgesia was not administered. Because untreated postoperative pain and stress may affect inflammatory and oxidative responses, the absence of postoperative analgesia represents both a methodological limitation and an animal-welfare concern. Future studies should incorporate an ethically appropriate standardized analgesic regimen while accounting for the potential effects of analgesic agents on oxidative, inflammatory, and bone healing outcomes [7, 8, 25].

Translational relevance

These findings may inform further investigation of locally delivered AgNPs in fracture repair, although translation should be approached cautiously. Increased callus formation, earlier radiographic progression, higher osteoblast and osteocyte counts, and changes in antioxidant enzyme measurements indicate that locally administered AgNPs altered the biological course of fracture healing in this model. AgNP-containing biomaterials have been investigated for orthopedic applications because their antimicrobial properties may potentially coexist with effects on tissue regeneration [22, 23]. Such combined properties could be relevant where infection risk and impaired bone repair occur concurrently.

Nevertheless, the present study evaluated only a single local administration in a rat femoral osteotomy model and therefore cannot establish clinical therapeutic efficacy. Furthermore, local administration does not necessarily eliminate systemic exposure because AgNPs or released Ag⁺ ions may migrate from the administration site into adjacent tissues or the systemic circulation [18, 35]. Clinical translation would therefore require systematic evaluation of local persistence, biodistribution, Ag⁺ release, tissue silver accumulation, systemic toxicity, and long-term effects on bone remodeling [36, 37].

Study limitations and future directions

Several limitations should be considered when interpreting these findings. First, larger radiographic callus measurements and higher histomorphometric cell counts in AgNP-treated groups do not necessarily indicate greater mechanical strength or functional recovery because biomechanical testing was not performed. Radiographic and histological progression does not necessarily establish restoration of normal mechanical properties; therefore, three-point bending or torsional testing should be incorporated into future studies [38].

Second, the authors did not quantify local silver concentration or tissue silver accumulation, limiting assessment of the relationship between the nominal administered concentration and actual biological exposure at the fracture site. Although the study detected Ag⁺ release from the preparation under the laboratory conditions described in the Methods, it did not separately evaluate its contribution to the observed biological responses. Third, the absence of direct measurements of reactive oxygen species and oxidative damage markers limited interpretation of the antioxidant findings. Fourth, osteogenic, angiogenic, and inflammatory molecular markers were not assessed; consequently, involvement of specific cytokine, vascular, macrophage, or osteogenic signaling pathways remains undetermined.

Fifth, the 30-day follow-up period did not permit evaluation of long-term remodeling, durability of the observed differences, or delayed local and systemic toxicity. Finally, the absence of postoperative analgesia represents a potential methodological confounder because postoperative pain and stress may influence inflammatory and oxidative responses [7, 8]. Future investigations should therefore include larger sample sizes, longer follow-up periods, standardized analgesia, biomechanical testing, local and systemic silver quantification, direct cytotoxicity and oxidative damage assessments, and molecular evaluation of osteogenic, inflammatory, angiogenic, and oxidative pathways.

Overall interpretation

Overall, a single local administration of AgNPs at 2.5, 5, and 10 µg/mL was associated with concentration- and time-dependent differences in fracture healing progression in the rat femoral osteotomy model. Among the evaluated concentrations, 10 µg/mL was associated with the largest radiographic callus size, higher osteoblast and osteocyte counts, and an earlier osteoclast response. AgNP administration was also associated with increased CAT and decreased SOD measurements, indicating an altered antioxidant enzyme profile.

These findings broadly align with previous evidence that AgNPs can influence bone-regenerative processes. However, differences in nanoparticle size, physicochemical characteristics, surface stabilization, concentration, delivery method, and experimental model must be considered when comparing studies [1, 6, 15, 17]. The present findings support further investigation of local AgNP administration as a potential adjunct to fracture repair but do not establish an optimal therapeutic concentration, mechanical superiority, long-term safety, or a specific molecular mechanism. Further preclinical studies integrating biomechanical, molecular, toxicological, and silver-distribution assessments are required before the therapeutic significance of these findings can be established.

CONCLUSION

A single local administration of AgNPs at 2.5, 5, and 10 µg/mL was associated with concentration- and time-dependent differences in fracture healing progression in the stabilized rat femoral osteotomy model. Among the evaluated concentrations, 10 µg/mL produced the largest radiographic callus size and was associated with higher osteoblast and osteocyte counts and an earlier osteoclast response. Histological findings were consistent with more advanced fracture repair in the AgNP-treated groups, particularly at the higher concentrations. AgNP administration was also associated with increased CAT and decreased SOD measurements, indicating an altered antioxidant enzyme profile rather than unequivocal enhancement of antioxidant defense. Collectively, the radiographic, histological, and histomorphometric findings indicate that local AgNP administration was associated with faster fracture repair progression under the conditions of this study.

The observed concentration-dependent differences support further investigation of locally administered AgNPs as a potential adjunct to fracture repair. Local delivery may provide direct exposure at the fracture site while potentially exploiting the biological and antimicrobial properties associated with AgNPs. However, these preclinical findings do not establish therapeutic efficacy, an optimal dose, or clinical safety. In particular, the pronounced healing-associated findings at 10 µg/mL coincided with the greatest changes in CAT and SOD measurements, underscoring the need to define an appropriate therapeutic window before considering translational applications.

The study evaluated three AgNP concentrations using a standardized, mechanically stabilized femoral osteotomy model and included complementary assessments of fracture repair at multiple postoperative time points. Serial radiographic measurements were supported by histological scoring and quantitative histomorphometric assessment of osteoblasts, osteocytes, and osteoclasts. Physicochemical characterization of the AgNP preparation further supported interpretation by defining key formulation characteristics, including primary particle size, hydrodynamic diameter, polydispersity, zeta potential, and crystalline structure. Assessment

of CAT and SOD provided additional information on changes in the antioxidant enzyme profile accompanying fracture repair.

Local AgNP administration accelerated radiographic, histological, and histomorphometric progression of fracture healing in this rat femoral osteotomy model, with the most pronounced healing-associated findings at 10 µg/mL. Nevertheless, changes in CAT and SOD, along with the lack of biomechanical, biodistribution, and long-term toxicological assessments, preclude identifying 10 µg/mL as an optimal or definitively safe therapeutic concentration. These findings support further preclinical investigation rather than immediate clinical application. Comprehensive biomechanical, molecular, toxicological, and pharmacokinetic evaluation is required to determine the efficacy, mechanism, therapeutic window, and long-term safety of local AgNP administration for fracture repair.

DATA AVAILABILITY

The data generated and analyzed during the current study are available from the corresponding author upon reasonable request.

GENERATIVE AI DECLARATION

During preparation of this manuscript, the authors used ChatGPT (OpenAI) for language editing and wording assistance. The authors reviewed and edited all AI-assisted content and take full responsibility for the accuracy and integrity of the final manuscript.

AUTHORS' CONTRIBUTIONS

SAM: Writing – original draft, statistical analysis, histopathological examination, and manuscript submission. OJA: Supervision, study design, and methodology. Both authors have read and approved the final version of the manuscript.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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