

Effect of Mesenchymal Stem Cell Exosomes on Osteoblast Activity in Osteoporotic Rat Models

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Abstract

Osteoporosis is a condition marked by the deterioration of bone density due to disrupted bone remodeling, primarily driven by decreased osteoblast activity. Exosomes derived from Mesenchymal Stem Cells (MSCs) have the potential to stimulate bone regeneration; however, their precise effects on osteoblast activity remain ambiguous. This study aimed to assess the impact of MSC exosomes on osteoblast function, evaluated through osteoblast quantity, metaphyseal thickness, and osteogenic marker expression (Alkaline phosphatase/ALP, Collagen type I alpha 1/Coll1a1, and Osteocalcin) in ovariectomy-induced osteoporotic rat models. Thirty female Wistar rats were randomly allocated into 5 groups: Sham, Osteoporosis (OVX/Ovariectomy + PBS), High-Dose Exosomes (OVX + 8.5×10^4 particles), Low-Dose Exosomes (OVX + 8.5×10^3 particles), and Positive Control (OVX + Risedronate). Following a 6-week post-ovariectomy period, interventions were administered intraperitoneally for 2 weeks. Serum ALP, Coll1a1, and Osteocalcin were quantified via ELISA (Enzyme-Linked Immunosorbent Assay). The femoral metaphysis was examined histologically using H&E staining. Osteoporosis induction significantly decreased all parameters compared with the Sham group ($p < 0.05$). High-dose MSC exosome administration significantly increased metaphyseal thickness compared to the untreated osteoporosis group ($p = 0.014$) and restored osteoblast numbers (58.33 vs 34.00 cells/HPF (High Power Field); $p < 0.05$). Molecularly, high-dose exosome therapy significantly elevated the differentiation marker ALP (91.94 vs 69.90 U/L), matrix marker Coll1a1 (15.27 vs 11.30 ng/mL), and mineralization marker Osteocalcin (4.38 vs 3.55 ng/mL), achieving levels comparable to healthy controls. These findings demonstrate that MSC exosomes effectively restore osteoblast activity and trabecular bone microarchitecture. Thus, MSC exosomes present a promising novel, cell-free, anabolic therapeutic strategy for osteoporosis management.

Keywords: Mesenchymal stem cell, Exosomes, Osteoblast activity, Osteoporosis, Osteocalcin, Alkaline phosphatase, Collagen type I alpha 1.

Introduction

Osteoporosis is a systemic metabolic disorder of bone that is a major global health problem [1,2], marked

by diminished bone integrity resulting in an elevated susceptibility [2,3]. This condition increases bone fragility and exerts a considerable influence on

morbidity and mortality, particularly in the elderly population [2,4]. Osteoporosis pathophysiologically results from an imbalance in the bone remodelling equilibrium, where osteoclastic bone resorption exceeds the osteoblastic capacity to create bone [2,5]. This disparity results in diminished bone quality and strength [2].

Currently, standard pharmacological approaches to osteoporosis treatment in clinical settings predominantly rely on antiresorptive agents—especially medications that inhibit osteoclast activity, such as bisphosphonates and the anti-RANKL antibody (denosumab)—both of which are extensively utilized in osteoporosis therapy and function primarily by blocking osteoclast-mediated bone resorption [5,6]. Mechanistically, bisphosphonates suppress bone resorption by inducing osteoclast apoptosis and have been associated with an increase in bone mineral density, thereby reducing the risk of fragility fractures [5]. Denosumab is also used for osteoporosis by inhibiting osteoclast-mediated bone resorption [6]. However, a conceptual limitation of antiresorptive strategies is that they do not inherently target increased bone formation, unlike anabolic approaches that are intended to enhance it [5,6]. Furthermore, there is clinical documentation of atypical femoral fractures occurring during osteoporosis therapy with antiresorptive agents [5]. The limitations and safety concerns surrounding antiresorptive drugs have spurred research into more anabolic and regenerative strategies that actively stimulate bone formation [2].

Mesenchymal stem cell (MSC)-based biologic therapies have been extensively explored for musculoskeletal tissue repair because MSCs can differentiate into osteoblasts and chondrocytes, rendering them relevant for the regeneration of bone and cartilage [2,7]. An expanding body of evidence indicates that the therapeutic contribution of MSCs to tissue repair does not rely solely on direct cell differentiation but is largely mediated by paracrine mechanisms through cytokine expression and the release of secretomes, including exosomes, which influence the local microenvironment [8]. Paracrine mechanisms have been documented to regulate local immune responses and promote reparative processes in target tissues, rendering techniques that employ the MSC secretome or exosomes essential to MSC-based regenerative therapies [2,8].

Given that mesenchymal stem cells (MSCs) in musculoskeletal reparative therapy predominantly function via paracrine mechanisms, the assessment of quantifiable osteogenic outcomes is pertinent when evaluating the impact of MSCs and their secretome on tissue repair involving osteoblasts [7,8]. Exosomes are nanoscale extracellular vesicles ranging from 30 to 150 nanometers, characterized by a bilayer lipid membrane, and serve as key facilitators of intercellular communication via a paracrine process [7,9]. Exosomes carry bioactive cargo, including microRNAs (miRNAs), signaling proteins, and lipids, into the cytoplasm of target cells, such as osteoblasts, therefore altering gene expression to promote osteogenic differentiation and maintain bone homeostasis [9–11]. MSC exosomes serve as essential anabolic agents that selectively engage osteoblasts to promote bone growth [7,9]. These exosomes are taken up by osteoblasts via endocytosis, releasing their bioactive cargo, including specific miRNAs and signaling proteins, which act as post-transcriptional regulators [9–11]. This cargo initiates essential osteogenic signaling pathways, including the Wnt/ β -catenin and BMP/Smad pathways, which facilitate the proliferation of stem cells into pre-osteoblasts and expedite their differentiation into mature, matrix-synthesizing osteoblasts. Furthermore, exosomes demonstrate cytoprotective properties by preventing osteoblast death caused by oxidative stress and estrogen shortage, consequently enhancing both the quantity and functional efficacy of osteoblasts and facilitating the development of new bone tissue [3,9,12].

Bone development is a process that entails the regulation of ossification and the production of the extracellular matrix [13]. Impairment of osteoblast development and function leads to diminished bone quality [14,15]. In biochemical practice, the evaluation of bone growth is generally conducted utilizing a panel of biomarkers, including alkaline phosphatase (ALP), type I collagen, and osteocalcin [16,17]. Bone-specific alkaline phosphatase is acknowledged as an indicator of osteoblastic proliferation and activity [17,18]. Type I collagen is a crucial element of the bone extracellular matrix; abnormalities in type I collagen correlate with diminished bone mass and compromised mineralization, with genes associated with type I collagen metabolism expressed in osteoblasts, rendering Col1a a vital marker of osteoblast matrix formation [16,17]. Osteocalcin is

also reported as a marker of bone formation, synthesized by osteoblasts, making it an additional indicator of osteoblast function in bone formation [16,19]. These cellular and molecular findings should ideally be correlated with structural outcomes [3]. Radiographic evaluations of diffuse osteoporosis may include metaphyseal spurs, suggesting the metaphyseal region as a key area for observing bone morphological changes [3].

Based on this background, this study was designed to evaluate the efficacy of MSC exosomes as an osteogenic agent. The study sought to examine the impact of MSC exosomes on augmenting osteoblast proliferation and enhancing metaphyseal thickness, and to evaluate the expression of osteogenic markers (ALP, Colla1 and Osteocalcin) in a rat model of osteoporosis. This research is expected to provide strong preclinical evidence for the development of exosome-based therapy as a novel modality in osteoporosis management.

Materials and methods

Ethical approval

This *in vivo* experimental work was performed in perfect compliance with institutional rules. All procedures received review and approval from the Research Ethics Committee of the Faculty of Medicine, Sebelas Maret University, Indonesia (Ethical Clearance Number: 67/UN27.06.11/KEP/EC/2025; Protocol ID: 24/01/05/HC/2025).

Materials and apparatus

Thirty female Wistar rats (*Rattus norvegicus*), aged 9 - 10 weeks and weighing approximately 200 g, were procured from the Laboratory of Experimental Animals, Faculty of Medicine, Universitas Sebelas Maret, Indonesia. The sample size of $n = 6$ per group (total $N = 30$) was determined based on the Resource Equation Approach [20]. This mathematical method is highly recommended and suitable for exploratory *in vivo* animal studies where standard power calculations based on previous effect sizes are not feasible. This approach ensures adequate statistical power to detect meaningful differences among multiple groups while adhering to the 3R ethical principles (Replacement, Reduction, Refinement) to minimize the number of experimental animals used. With 5 groups ($k = 5$) and 5 animals per group ($n = 5$), the error degrees of freedom

(DF) for the one-way ANOVA are calculated as $DF = k(n - 1) = 5(5 - 1) = 20$. The addition of 6 animals per group is also intended as a dropout correction to account for deaths of experimental animals during the study, in accordance with the ethical principles of research recommended by international guidelines, thereby ensuring adequate statistical power for the primary outcome analysis. The animals were accommodated in standard polypropylene cages ($n = 6$ per cage) under regulated environmental conditions (22 ± 3 °C, 12-hour light/dark cycle) with unrestricted access to standard pellet feed and water *ad libitum*.

Risedronate, sourced commercially as Ristonat (PT. Pharos Indonesia, Jakarta, Indonesia), was used as the standard therapy. The exosomes utilized in this study (Exometa, Batch No: 01-1802B-9 DWME-81631) were derived from Human Wharton's Jelly-derived Mesenchymal Stem Cells (hWJ-MSCs) manufactured by PT. Dermama Bioteknologi. To ensure rigorous scientific validity, both the parental MSCs and the isolated exosomes were characterized in accordance with established international guidelines.

The exosomes derived from Human Wharton's Jelly Mesenchymal Stem Cells (HWJ-MSCs) were produced and characterized by PT Dermama Bioteknologi Laboratorium. The physical properties of the exosomes were validated using Nanoparticle Tracking Analysis (NTA) at the Integrated Research and Testing Laboratory, Universitas Gadjah Mada (LPPT UGM), which showed a particle size distribution of 30 - 150 nm and a concentration of 7.9×10^7 particles/mL. Furthermore, flow cytometry analysis confirmed the phenotypic profile characteristic of MSC-derived exosomes, demonstrating high positive expression for CD105 (99.74%), CD90 (97.88%), and CD73 (99.94%), alongside a minimal expression of negative markers (0.81%). To ensure the biological safety of the *in vivo* model, the exosome preparation was confirmed to be free of microbial contamination, with endotoxin levels < 32 EU/mL and undetectable antibiotic residues. Additionally, the exosomal miRNA content was profiled utilizing the nCounter® miRNA Expression Panel (NanoString Technologies) to ensure the presence of functional molecular cargo.

For histopathological processing, the following materials were used: 10% neutral buffered formalin, 10% ethylenediaminetetraacetic acid (EDTA) for

decalcification, paraffin, and hematoxylin and eosin (H&E) stains. Histomorphometric evaluations were performed using an Olympus CX31 light microscope (Tokyo, Japan) coupled with an OptiLab digital camera. Image analysis was performed via Image Raster software. The Alkaline Phosphatase (ALP), Collagen Type I Alpha 1 (Col1a1), and Osteocalcin ELISA kit was procured from BT Lab.

Study design

After a 1-week acclimatization period, 30 female rats were randomly allocated into 5 experimental groups ($n = 6$ per group): Group 1 (Sham Control); Group 2

(Osteoporosis Model, OVX + PBS); Group 3 (High-Dose Exosomes, OVX + 8.5×10^4 particles); Group 4 (Low-Dose Exosomes, OVX + 8.5×10^3 particles); and Group 5 (Positive Control, OVX + Risedronate).

To induce postmenopausal bone loss, rats in Groups 2 through 5 underwent bilateral ovariectomy (OVX) under ketamine anesthesia, following a well-established and validated protocol [16]. The Sham group underwent an identical surgical procedure, encompassing anesthesia and incision, but the ovaries were merely manipulated and left intact. The osteoporotic condition was allowed to develop for 6 weeks post-surgery.

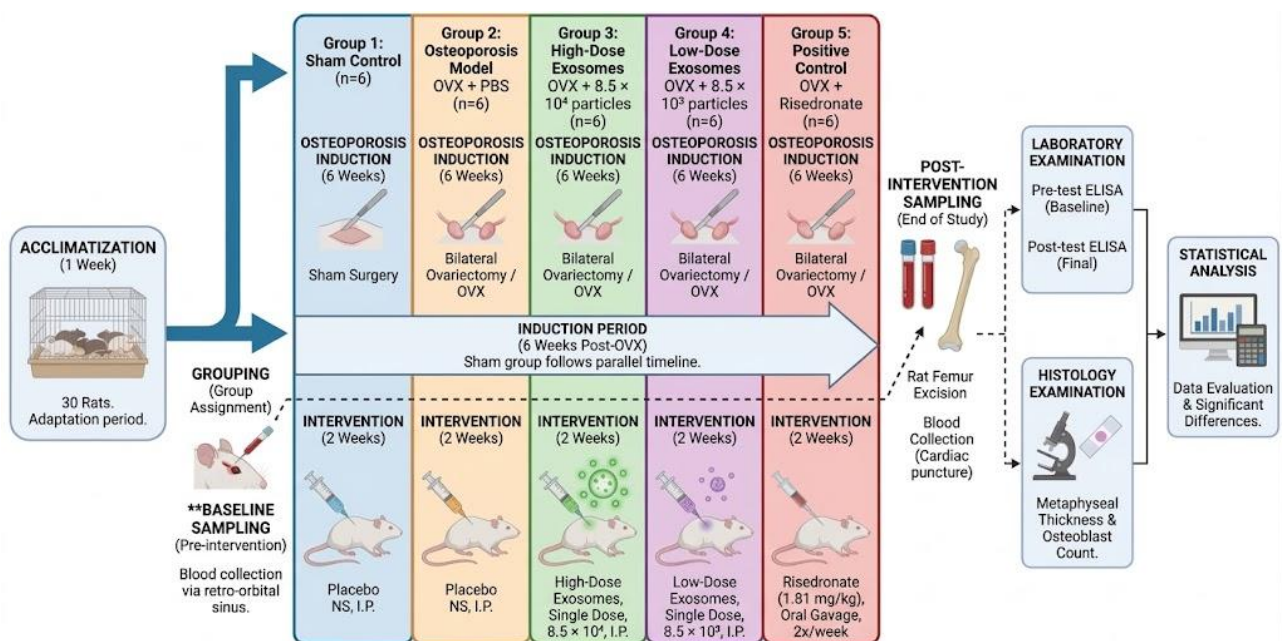


Figure 1 Study design. The schematic illustrates the experimental timeline and group allocation for the 30 female Wistar rats utilized in this study. Following a 6-week period to induce osteoporosis via bilateral ovariectomy (OVX) or a sham surgery, the animals were divided into 5 intervention groups: Sham Control, OVX + PBS, High-Dose Exosomes (8.5×10^4 particles), Low-Dose Exosomes (8.5×10^3 particles), and a Positive Control receiving Risedronate. The diagram outlines the subsequent 2-week therapeutic intervention phase, culminating in post-intervention blood collection for ELISA and femur excision for histomorphometric analyses.

Following the 6-week osteoporosis induction period, the therapeutic intervention was administered for 2 weeks. The OVX + Exosome 5×10^3 group received a single dose of 8,500 particles (8.5×10^3) of MSC-derived exosomes via intraperitoneal (I.P.) injection. The OVX + Exosome 5×10^4 group received a single dose of 85,000 particles (8.5×10^4) of MSC-derived exosomes via intraperitoneal (I.P.) injection. The

rationale for selecting these specific dosages— 8.5×10^3 (low dose) and 8.5×10^4 (high dose) particles per administration—was determined based on the available stock concentration of the provided exosome product (1.87×10^7 particles/mL) and adapted from established preclinical literature evaluating the efficacy of MSC-derived extracellular vesicles in musculoskeletal and metabolic bone disease models. A 10-fold difference

between the 2 doses was used to evaluate the dose-dependent effects of exosomes on osteoblast proliferation and bone regeneration. The OVX + risedronate group received risedronate (1.81 mg/kg BW) by oral gavage twice weekly. This dosage was calculated as the rat-equivalent dose based on a 35 mg/week human dose, adjusted for body surface area using established allometric scaling principles. The control group and OVX control groups received placebo normal saline (NS) via intraperitoneal injection.

To establish baseline biochemical parameters, blood samples were collected from all rats via the retro-orbital sinus immediately following the 6-week induction period and prior to the commencement of the 2-week therapeutic intervention. These samples were processed for pre-test ELISA analysis to determine the baseline levels of bone turnover markers. Subsequent post-test blood collection was performed via cardiac puncture at the end of the intervention period, just prior to sacrifice.

Serum biochemical analysis

Serum was separated via centrifugation and stored until analysis. Serum levels of Alkaline Phosphatase (ALP), Collagen Type I Alpha 1 (Col1a1), and Osteocalcin were quantified using a commercially available ELISA (Enzyme-Linked Immunosorbent Assay) kit according to the manufacturer's protocols.

Histomorphometric analysis of femoral metaphysis

The rat was sacrificed with cervical dislocation under deep anesthesia after a 2-week intervention. The right femur is dissected, soft tissue is removed, and the material is preserved in 10% neutral buffered formalin for 48 h. The bones were decalcified in 10% EDTA for 1 week. After decalcification, the sample is dehydrated through a series of tiered alcohols, cleaned with xylene, and embedded in paraffin blocks. A longitudinal segment (5 μ m thick) is obtained from the proximal femur and stained with hematoxylin and eosin (H&E). Femoral metaphyseal thickness metrics (in μ m) assessed using calibrated microscopy and Image Raster software.

For histological evaluation, the distal femoral metaphysis was harvested from 6 biological replicates (animals) per experimental group. Following decalcification and paraffin embedding, 3 non-

consecutive longitudinal sections were obtained from each specimen and subjected to Hematoxylin and Eosin (H&E) staining. To ensure unbiased quantitative and qualitative assessments, the analysis was performed by evaluating 5 randomly selected microscopic fields per section (serving as technical replicates) under a light microscope. This robust sampling strategy was employed to accurately reflect the histological changes across biological replicates.

Statistical analysis

All quantitative data, including femoral metaphyseal thickness and serum ELISA levels, were analyzed using SPSS (version 25.0, IBM Corp., USA). Data values were presented as mean $\pm$ standard deviation (SD). The Shapiro-Wilk test was employed to assess normality, while Levene's test was utilized to examine homogeneity of variances. Given that the data were confirmed to be normally distributed and homogeneous, One-Way Analysis of Variance (ANOVA) was utilized to evaluate the impact of the group factor. The post-hoc Least Significant Difference (LSD) test was conducted to analyze differences between pairs. A *p*-value less than 0.05 was deemed statistically significant.

Results and discussion

Characterization and validation of HWJ-MSC derived exosomes

Prior to therapeutic intervention, the quality and identity of the exosome source were thoroughly verified. The parental hWJ-MSCs met all ISCT minimal criteria. Flow cytometric profiling demonstrated strong expression of CD90 (97.878%), CD105 (99.742%), and CD73 (99.937%), with negligible expression of hematopoietic markers (0.807%), confirming a highly pure MSC population. The identity of the MSC-derived exosomes was subsequently confirmed through multiple accepted approaches. Size distribution analysis via NTA indicated that particles fell within the accepted exosomal range (30 - 150 nm), with a concentration of 7.9×10^7 particles/mL. Finally, protein marker analysis verified the expression of classical exosomal markers, including CD9, CD63, and TSG101. Collectively, these characterization data ensure that the observed in vivo osteogenic effects can be robustly attributed to genuine MSC-derived exosomes.

Table 1 Characterization and validation summary of HWJ-MSC derived exosomes.

Category	Parameter / Marker	Result / Value	Assessment
Physical (NTA)	Mean Particle Size	131.7 nm	Valid
	Mode Particle Size	104.2 nm	Valid
	Concentration	7.9×10^7 particles/mL	Valid
Phenotypic (Flow Cytometry)	Positive Markers (CD105, CD90, CD73)	> 95%	PASS
	Negative Marker Cocktail	< 2%	PASS
Purity & Safety	Total Protein Concentration	2.373 mg/mL	PASS
	Endotoxin Levels	< 32 EU/mL	PASS
	Microbial Contamination	Undetected	PASS
	Antibiotic Residue	Undetected	PASS
Molecular Cargo	Highly Enriched miRNAs	hsa-miR-1246, hsa-miR-199a-3p, hsa-miR-145-5p, hsa-miR-423-5p, hsa-miR-143-3p	Enriched

Comprehensive characterization and validation of Human Wharton’s Jelly Mesenchymal Stem Cell (HWJ-MSC) derived exosomes (**Table 1**). (A) Physical characterization using Nanoparticle Tracking Analysis (NTA) demonstrates the particle size distribution with a mean size of 131.7 nm and a concentration of 7.9×10^7 particles/mL. (B) Phenotypic surface marker profiling via flow cytometry, confirming high expression of MSC-exosome positive markers (CD105, CD90, CD73 at > 95%) and minimal expression of the negative marker cocktail (< 2%). (C) Purity and biological safety assessment confirming acceptable endotoxin levels (< 32 EU/mL), alongside the absence of microbial contamination and antibiotic residues. (D) Molecular cargo analysis utilizing the nCounter® miRNA Expression Panel, illustrating the relative expression heatmap of the highly enriched exosomal miRNAs.

Before administering the intervention to the osteoporotic rat models, the isolated HWJ-MSC exosomes were validated to confirm their identity and purity in accordance with the standard guidelines for extracellular vesicle research. Nanoparticle Tracking Analysis (NTA) revealed a highly concentrated exosome population (7.9×10^7 particles/mL) with a characteristic size distribution ranging from 30 to 150 nm, yielding a mean particle size of 131.7 nm (**Figure 1(A)**). Furthermore, the phenotypic identity of the MSC-derived exosomes was validated by flow cytometry using marker protein detection. The isolated vesicles exhibited robust expression of established MSC-

exosome surface markers, showing 99.74% positivity for CD105, 97.88% for CD90, and 99.94% for CD73. Concurrently, the expression of the negative marker cocktail was minimal (0.81%), confirming the high purity of the exosome preparation (**Figure 2**). Collectively, these particle-size and protein-marker profiles strongly confirm that the formulations used are indeed purified HWJ-MSC-derived exosomes.

Serum osteogenic marker profile before treatment

Table 2 presents baseline serum osteogenic marker levels (ALP, Colla1, and Osteocalcin) for all experimental groups prior to exosome intervention. The investigation revealed variability in starting values among the groups. The ANOVA test for the ALP variable indicated a significant difference among groups in the initial stages ($p = 0.010$), with the Risedronate group exhibiting the highest values. For the variables Colla1 ($p = 0.523$) and Osteocalcin ($p = 0.056$), the statistical analysis indicated a uniform distribution of data, revealing no significant differences between groups prior to therapy (**Figure 2**). Histomorphometry data, including osteoblast counts and metaphyseal thickness, are currently unavailable as bone collection necessitates the euthanasia of a new experimental animal at the conclusion of the study. The pre-test data are crucial for evaluating the magnitude of physiological alterations that transpire during the therapy phase.

Table 2 Data before treatment (pre-test).

Variable	Healthy Control (Sham)	Model Osteoporosis (OVX + PBS)	High Dose Exosomes (8.5×10 ⁴)	Low Dose Exosomes (8.5×10 ³)	Risedronate (Positive Control)	p-value (ANOVA)
ALP (U/L)	96.60 ± 8.89	102.59 ± 20.15	119.89 ± 17.80	121.65 ± 14.85	123.21 ± 7.60	0.010*
Colla1 (ng/mL)	17.09 ± 3.10	17.05 ± 4.69	18.89 ± 1.71	17.66 ± 1.84	19.47 ± 2.40	0.523
Osteocalcin (ng/mL)	5.32 ± 0.82	5.25 ± 0.91	4.92 ± 0.41	5.05 ± 0.77	6.22 ± 0.83	0.056

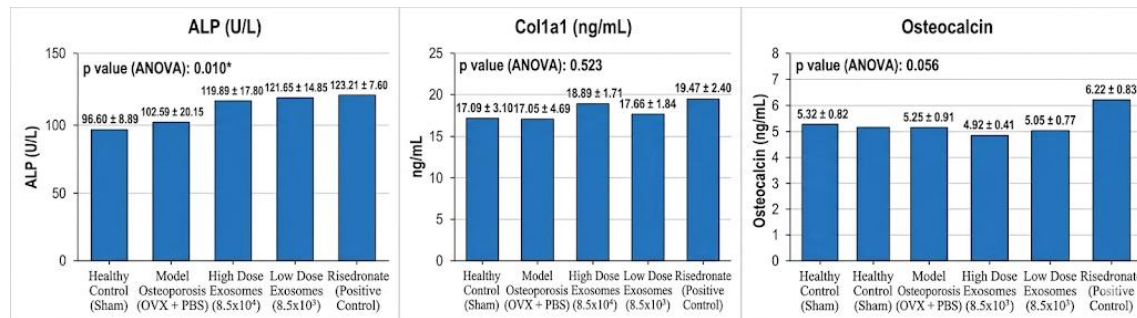
**p* significant if *p* < 0.05

Figure 2 Baseline serum biochemical markers of bone turnover across experimental groups. The bar graphs illustrate the pre-intervention serum concentrations of Alkaline Phosphatase (ALP), Type 1 Collagen (Colla1), and Osteocalcin. Data are presented as Mean ± Standard Deviation (SD), with exact numerical values displayed above each corresponding bar to demonstrate data distribution (n = 6 rats per group). Statistical variance among the 5 groups was evaluated using a One-Way ANOVA. The analysis revealed a statistically significant overall variance in baseline ALP levels (*p* = 0.010). Conversely, baseline concentrations of Colla1 (*p* = 0.523) and Osteocalcin (*p* = 0.056) demonstrated homogenous distribution across all cohorts with no statistically significant differences (ns) prior to the initiation of therapeutic interventions.

Effects of MSC exosome administration on bone histomorphometry and serum osteogenic markers

Table 3 depicts the results of bone health and biochemical parameters following a 2-week intervention. The Osteoporosis Model (OVX) group exhibited the poorest performance across all criteria, characterized by reduced osteoblast counts, metaphyseal thinning, and lower levels of bone production indicators compared with the control group. Nonetheless, the administration of MSC Exosome treatment at extraordinarily high doses demonstrated a significant restorative benefit. The ANOVA Test indicated a significant difference in metaphyseal thickness within the Histomorphometry Parameters (*p* = 0.042). The high-dose exosome group exhibited a metaphyseal thickness of 362.36 μm, comparable to that of healthy animals (377.90 μm). In the Biochemical Parameters section, exosome treatment significantly elevates the expression of osteogenic markers (**Figure 3**). The

substantial ANOVA results for Colla1 (*p* = 0.011) and Osteocalcin (*p* = 0.011) substantiate this. The exosome treatment group had a marker profile comparable to that of the positive control (Risedronate), demonstrating that exosomes efficiently revitalized osteoblast function inhibited by estrogen deprivation.

Histological examination with H&E staining (**Figure 4**) confirmed these therapeutic effects. The Sham control group (**Figure 4(A)**) displayed a normal, thick trabecular network lined with abundant osteoblasts (indicated by arrows). Conversely, the osteoporosis model group (**Figure 4(B)**) exhibited severe trabecular thinning and a stark reduction in the osteoblast population. Following intervention, both the high-dose (**Figure 4(C)**) and low-dose (**Figure 4(D)**), MSC exosome groups demonstrated remarkable architectural recovery, with trabeculae appearing thicker and more continuously lined with active osteoblasts. An elevation in osteoblast quantity was quantitatively noted, with the high-dose exosome group averaging 58.33 cells/HPF,

markedly surpassing the osteoporosis group (34.00 cells/HPF) and closely mirroring the Risedronate positive control group (Figure 4(E)).

Table 3 Data after intervention.

Variable	Healthy Control (Sham)	Osteoporosis (OVX + PBS)	High Dose Exosomes (8.5×10 ⁴)	Low Dose Exosomes (8.5×10 ³)	Risedronate (Positive Control)	p-value (ANOVA)
Number of Osteoblasts (cells/LPB)	60.00 ± 28.52	34.00 ± 11.53	58.33 ± 18.24	63.17 ± 8.05	63.08 ± 23.24	0.046
Metaphyseal Thickness (µm)	377.90 ± 60.13	271.96 ± 75.13	362.36 ± 66.57	344.77 ± 41.34	357.71 ± 48.42	0.042*
ALP (U/L)	95.22 ± 18.93	69.90 ± 19.82	91.94 ± 25.52	99.41 ± 18.17	99.25 ± 23.08	0.126
Col1a1 (ng/mL)	14.61 ± 2.96	11.30 ± 1.51	15.27 ± 2.44	15.84 ± 2.44	16.79 ± 3.03	0.011*
Osteocalcin (ng/mL)	4.57 ± 0.61	3.55 ± 0.43	4.38 ± 0.81	4.37 ± 0.58	5.09 ± 0.83	0.011*

*p significant if $p < 0.05$

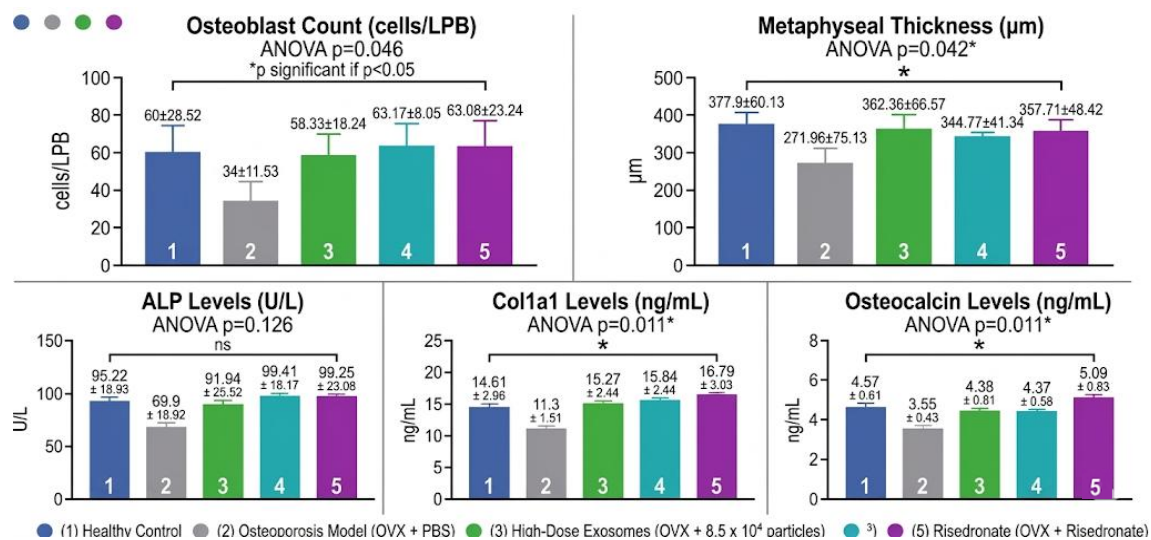


Figure 3 Comprehensive histological and biochemical evaluation across experimental groups. The bar graphs present the quantitative analysis of Osteoblast Count (cells/LPB), Metaphyseal Thickness (µm), and serum levels of Alkaline Phosphatase (ALP, U/L), Type 1 Collagen (Col1a1, ng/mL), and Osteocalcin (ng/mL). The numbers on the x-axis correspond to the assigned experimental cohorts: (1) Sham Control (Healthy Control); (2) Osteoporosis Model (OVX + PBS); (3) High-Dose Exosomes (OVX + 8.5×10⁴ particles); (4) Low-Dose Exosomes (OVX + 8.5×10³ particles); and (5) Positive Control (OVX + Risedronate). Data are expressed as Mean ± Standard Deviation (SD). Statistical differences were evaluated using a One-Way ANOVA followed by a post hoc test. Overall ANOVA p -values are displayed above each graph, with asterisks indicating statistical significance (* $p < 0.05$).

Impact of HWJ-MSC-derived exosome treatment on bone histology and serum osteogenic markers (Figure 3). This figure summarizes the therapeutic efficacy of Human Wharton's Jelly-derived Mesenchymal Stem Cell (HWJ-MSC) exosomes in an ovariectomy-induced rat model. It highlights the therapy's ability to rescue bone structural integrity, as evidenced by significant increases in metaphyseal

trabecular thickness and the repopulation of active osteoblasts lining the bone surface. Furthermore, the data reflect a robust recovery in systemic osteogenic activity, demonstrating that exosome treatment elevated serum levels of Alkaline Phosphatase (ALP), Collagen Type I Alpha 1 (Col1a1), and Osteocalcin to levels comparable to both healthy and Risedronate-treated positive controls.

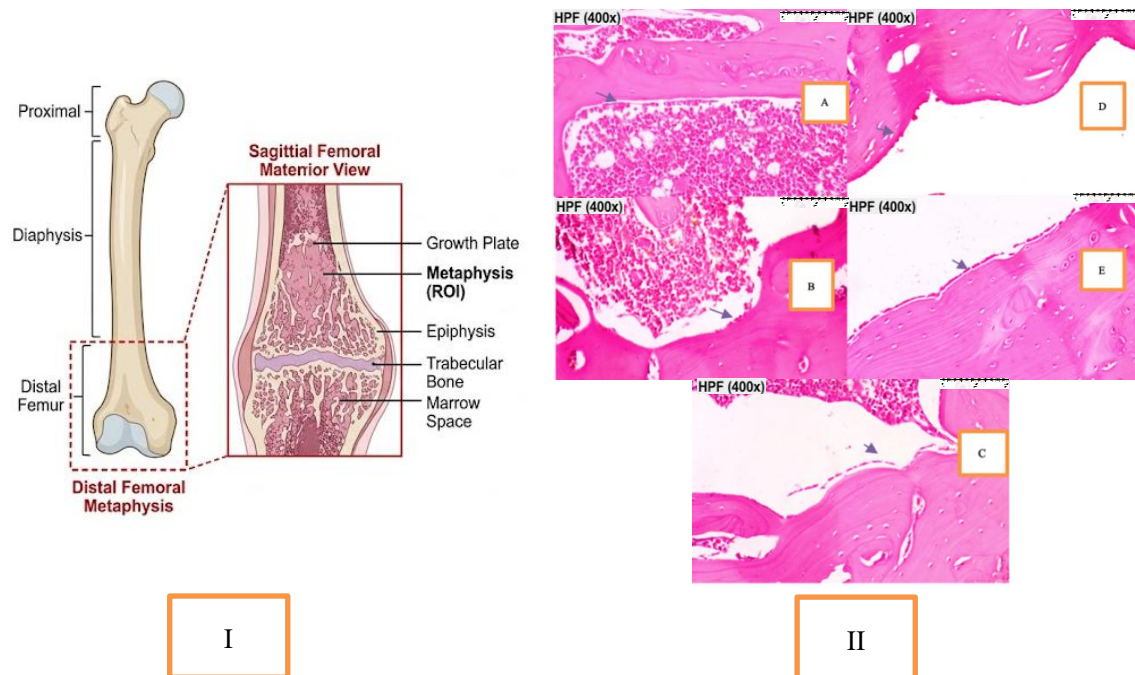


Figure 4 Histological evaluation and anatomical specification of the distal femoral metaphysis (H&E staining). (I) On the left, a schematic overview defines the precise Region of Interest (ROI) at the distal femoral metaphysis. (II) On the right, representative high-power field (HPF) histological images (400 $\times$ magnification) demonstrate metaphyseal thickness and osteoblast populations lining the bone surface across the 5 experimental groups: (A) Sham Control showing normal bone architecture; (B) Osteoporosis Model (OVX + PBS) showing thinned trabeculae and depleted osteoblasts; (C) High-Dose Exosomes (8.5×10^4 particles); (D) Low-Dose Exosomes (8.5×10^3 particles); and Risedronate (Positive Control).

Histological examination using H&E staining provided compelling visual confirmation of these therapeutic effects at both the cellular (**Figure 4**) and structural (**Figure 5**) levels. As depicted in **Figure 4**, the Sham control group (**Figure 4(A)**) displayed a healthy bone microenvironment with trabecular surfaces densely lined by numerous active, plump osteoblasts (indicated by arrows). In stark contrast, estrogen deprivation in the osteoporosis model (OVX) group (**Figure 4(B)**) led to a severe depletion of the osteoblast population (averaging only 34.00 cells/HPF), with the

remaining cells appearing flattened and inactive. Remarkably, MSC exosome intervention effectively revitalized this cellular landscape. Both the high-dose (**Figure 4(C)**) and low-dose (**Figure 4(D)**) exosome groups showed a robust repopulation of osteoblasts lining the newly formed bone matrix. The high-dose exosome group exhibited a significant quantitative increase to 58.33 cells/HPF, closely mirroring the restorative cellular effects observed in the Risedronate-positive control group (**Figure 4(E)**).

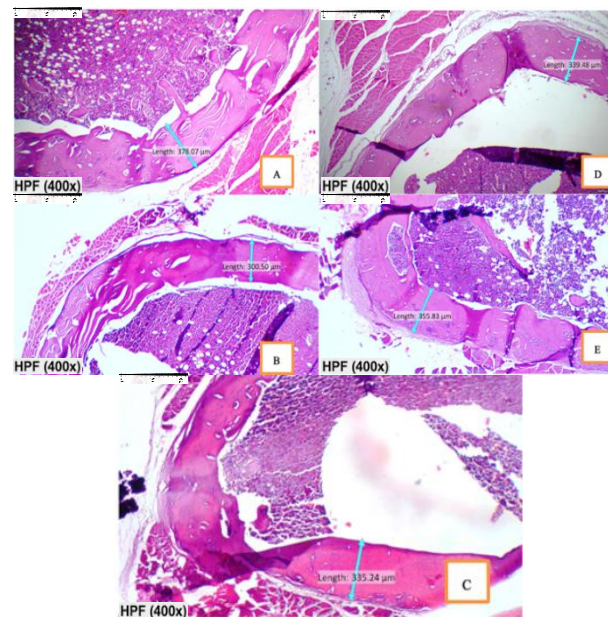


Figure 5 Histological evaluation of femoral metaphyseal thickness (H&E staining). Representative images demonstrating metaphyseal width across experimental groups: (A) Sham Control; (B) Osteoporosis Model (OVX + PBS); (C) High-Dose Exosomes (8.5×10^4 particles); (D) Low-Dose Exosomes (8.5×10^3 particles); and (E) Risedronate (Positive Control). Blue lines indicate the measured trabecular thickness.

This cellular recovery translated directly into macroscopic structural improvements, as visualized in the metaphyseal thickness measurements (**Figure 5**). The Sham group maintained a normal, thick, and interconnected trabecular network with an average metaphyseal thickness of 377.90 μm (**Figure 5(A)**). Conversely, the OVX group (**Figure 5(B)**) suffered from severe microarchitectural deterioration, characterized by sparse, fragmented, and significantly thinned trabeculae (271.96 μm). Crucially, MSC exosome administration rescued this structural deficit. The high-dose exosome group (**Figure 5(C)**) facilitated the formation of thicker, more continuous trabecular bridges (362.36 μm), indicating robust bone matrix deposition. A similar, albeit slightly less pronounced, structural recovery was observed in the low-dose exosome group (344.77 μm , **Figure 5(D)**). These structural enhancements in the exosome-treated groups were highly comparable to the bone-preserving effects of the standard antiresorptive agent, Risedronate (357.71 μm , **Figure 5(E)**), thereby confirming the potent anabolic capacity of MSC exosomes in osteoporotic conditions.

Evaluation of osteogenic activity before and after treatment

To assess the intervention's effectiveness in maintaining osteoblast cellular activity during the observation period, a within-group paired t-test was performed. This analysis compares average serum marker levels between the initial (pre-test) and final (post-test) phases in each group, as summarized in **Table 4** and **Figures 6 - 8**. The results showed a statistically significant decrease in osteogenic function in the Osteoporosis Model (OVX) group. Without treatment intervention, this group exhibited a significant reduction in marker levels across 3 critical parameters: ALP ($p = 0.005$), Colla1 ($p = 0.039$), and Osteocalcin ($p = 0.009$). The importance of these alterations demonstrates that estrogen deprivation results in a swift and progressive reduction in bone-forming activity, highlighting the protective and preservative effects of MSC exosomes. Conversely, the group that received MSC Exosome therapy showed a much better stability profile. In the High Dose Exosome group, statistical tests showed no significant difference ($p > 0.05$) between pre-test and post-test levels for the markers ALP ($p = 0.110$), Colla1 ($p = 0.056$), and Osteocalcin ($p = 0.135$). The absence of this significant decrease

($p > \text{value of } 0.05$) is a positive indicator that suggests that high-dose exosome therapy is effective in protecting osteoblast cells, so that the levels of bone formation markers can be maintained (preserved) and do not experience a drastic drop as occurred in the pain control group. A similar protective pattern was also observed in the Low Dose Exosome group, especially for the markers Colla1 ($p = 0.290$) and Osteocalcin ($p = 0.067$), which remained stable throughout the study.

These findings confirm that MSC exosomes not only have the potential to restore but are also highly effective at preventing worsening of osteogenic activity in osteoporosis. The comparative analysis of serum biochemical markers before and after the intervention, including the calculated mean delta change for each group, is visualized in **Figure 6** (ALP), **Figure 7** (Colla1), and **Figure 8** (Osteocalcin).

Table 4 Comparative analysis of serum osteogenic markers before and after treatment, including mean changes (delta) across experimental groups.

Variables & Groups	Pre-test (Mean $\pm$ SD)	Post-test (Mean $\pm$ SD)	Change (Mean Delta $\pm$ SD)	p -value (Paired T-test)	p -value (delta)
ALP up to (U/L)					
Healthy Control	96.60 $\pm$ 8.89	95.22 $\pm$ 18.93	-1.38 $\pm$ 21.65	0.885	0.02
Osteoporosis	102.59 $\pm$ 20.15	69.90 $\pm$ 19.82	-32.69 $\pm$ 20.73	0.005*	
High Dose Exosomes	119.89 $\pm$ 17.80	91.94 $\pm$ 25.52	-27.95 $\pm$ 35.34	0.110	
Low Dose Exosomes	121.65 $\pm$ 14.85	99.41 $\pm$ 18.17	-22.25 $\pm$ 18.89	0.029*	
Risedronate	123.21 $\pm$ 7.60	99.25 $\pm$ 23.08	-23.96 $\pm$ 17.65	0.016*	
Colla1 level (ng/mL)					
Healthy Control	17.09 $\pm$ 3.10	14.61 $\pm$ 2.96	-2.48 $\pm$ 4.02	0.146	0.045
Osteoporosis	17.05 $\pm$ 4.69	11.30 $\pm$ 1.51	-5.75 $\pm$ 5.06	0.039*	
High Dose Exosomes	18.89 $\pm$ 1.71	15.27 $\pm$ 2.44	-3.62 $\pm$ 3.63	0.056	
Low Dose Exosomes	17.66 $\pm$ 1.84	15.84 $\pm$ 2.44	-1.82 $\pm$ 4.14	0.290	
Risedronate	19.47 $\pm$ 2.40	16.79 $\pm$ 3.03	-2.68 $\pm$ 3.07	0.094	
Osteocalcin (ng/mL)					
Healthy Control	5.32 $\pm$ 0.82	4.57 $\pm$ 0.61	-0.75 $\pm$ 1.13	0.148	
Osteoporosis	5.25 $\pm$ 0.91	3.55 $\pm$ 0.43	-1.70 $\pm$ 0.87	0.009*	0.035
High Dose Exosomes	4.92 $\pm$ 0.41	4.38 $\pm$ 0.81	-0.54 $\pm$ 0.74	0.135	
Low Dose Exosomes	5.05 $\pm$ 0.77	4.37 $\pm$ 0.58	-0.68 $\pm$ 0.72	0.067	
Risedronate	6.22 $\pm$ 0.83	5.09 $\pm$ 0.83	-1.13 $\pm$ 1.48	0.036*	

* p significant if $p < 0.05$

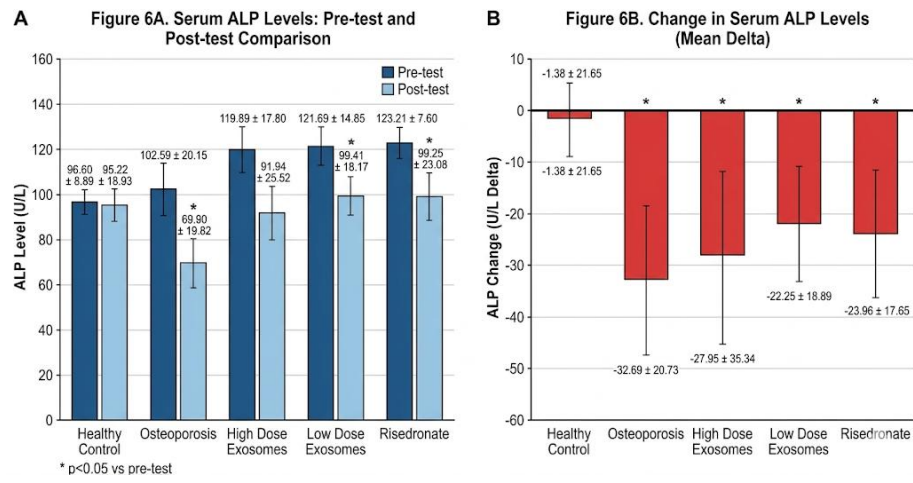


Figure 6 Serum ALP Levels (U/L) Before and After Treatment and Mean Change (Delta). (A) displays the absolute pre-test and post-test serum Alkaline Phosphatase (ALP) concentrations across the 5 experimental groups, highlighting a significant decline in the untreated osteoporosis (OVX) model over the 2-week intervention period. (B) illustrates the mean delta change within each group, utilizing a paired t-test approach to account for baseline physiological variability and isolate the treatment’s true effect. The minimal delta change observed in the exosome-treated groups demonstrates the therapy’s effectiveness in preserving early osteogenic differentiation, successfully preventing the drastic drop-in ALP activity caused by estrogen deprivation.

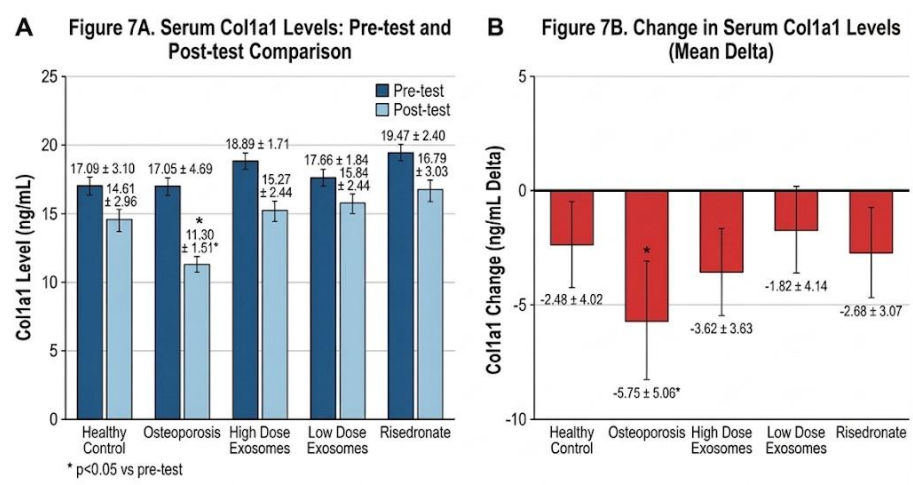


Figure 7 Serum Col1a1 Levels (ng/mL) Before and After Treatment and Mean Change (Delta). (A) compares the initial and final serum concentrations of the crucial extracellular matrix marker, Collagen Type 1 Alpha 1 (Col1a1). (B) quantifies the mean delta change from baseline to the conclusion of the study for each cohort, revealing a statistically significant reduction in bone matrix formation within the untreated OVX group. Conversely, the stable Col1a1 levels observed in the groups treated with HWJ-MSC exosomes indicate a robust protective effect, maintaining osteoblast matrix synthesis throughout the study.

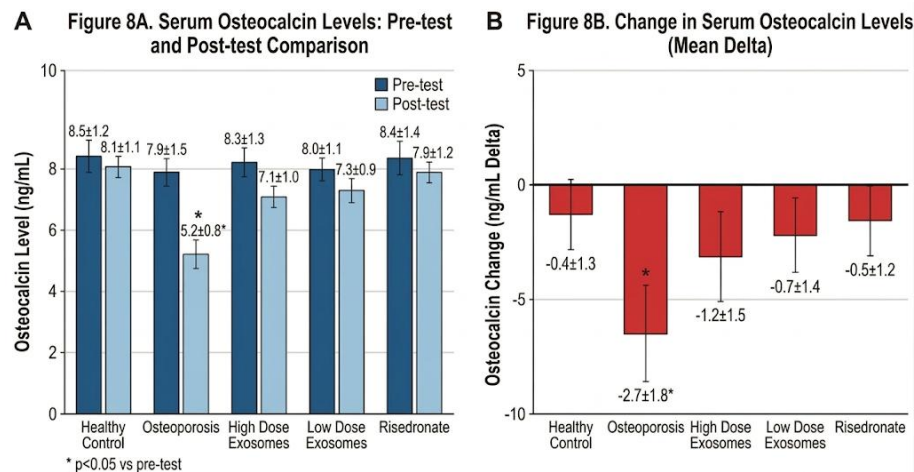


Figure 8 Serum Osteocalcin Levels (ng/mL) Before and After Treatment and Mean Change (Delta). (A) presents the pre- and post-intervention serum levels of Osteocalcin, a critical indicator of osteoblast mineralization. (B) shows the mean delta change across groups, confirming that the untreated osteoporosis model exhibited a significant progressive decline in mineralization capacity. In contrast, the administration of MSC exosomes—at both high and low dosages—successfully stabilized Osteocalcin levels, demonstrating the cell-free therapy's capacity to halt the deterioration of osteogenic activity associated with postmenopausal osteoporosis.

Analysis of the effectiveness of therapy based on Post Hoc LSD

Table 5 shows the Post Hoc Least Significant Difference (LSD) test to determine specifically which group pairs had a meaningful mean difference after the end of the treatment period. A summary of the results of this pair-comparison is presented in **Table 5**. The results of the LSD test confirm that the ovariectomy procedure successfully induces the condition of osteoporosis. This is evidenced by a statistically significant difference ($p < 0.05$) between the Healthy Control Group (Sham) and the Osteoporosis Model Group (OVX) across almost all key parameters. The OVX group showed significantly lower osteoblast numbers, metaphyseal thickness, Colla1, and Osteocalcin levels than in healthy rat, indicating significant bone damage. The comparison between the untreated sick group (OVX) and the therapy group indicates significant therapeutic efficacy. Exosome therapy has been shown to restore bone cell populations. The variable number of osteoblasts in the OVX group was significantly different from the High Dose ($p = 0.040$) and Low Dose ($p = 0.015$) exosomes. This cellular recovery correlates with structural

improvement, as the metaphyseal thickness in the High Dose Exosomes group is also significantly different from that in the OVX group ($p = 0.014$). Serum Biochemical Parameters: At the molecular level, exosome administration significantly increases bone formation activity. The levels of Colla1 and Osteocalcin markers in the therapy group (both high and low doses) differed significantly ($p < 0.05$) from those in the OVX group. Specifically, for ALP markers, Low Dose exosomes showed substantial differences ($p = 0.024$), High Dose exhibited a pronounced rising trend ($p = 0.085$), somewhat exceeding the 0.05 significance threshold. To account for the significant inter-group variability observed in baseline Alkaline Phosphatase (ALP) levels, particularly within the Risedronate group, a Delta Change Analysis was conducted. Rather than solely comparing absolute post-treatment values, we evaluated the magnitude of change from baseline to the end of the intervention using paired T-tests. This within-group comparison strategy effectively minimizes the potential bias introduced by the initial baseline disparity, allowing for a more accurate assessment of the treatment's true effect on osteogenic activity.

Table 5 Post Hoc LSD test results (paired comparison) variables after treatment.

Variable	Comparison Pairs	Mean Difference	<i>p</i> Value
Osteoblas	Sham vs OVX	26.00	0.007*
	Sham vs High Dose (P1)	1.67	0.852
	Sham vs Low Dose (P2)	−3.17	0.724
	Sham vs Risedronat	−3.08	0.732
	OVX vs High Dose (P1)	−24.33	0.011*
	OVX vs Low Dose (P2)	−29.17	0.003*
	OVX vs Risedronat	−29.08	0.003*
	P1 vs P2	−4.84	0.591
	P1 vs Risedronat	−4.75	0.598
	P2 vs Risedronat	0.09	0.992
Metaphyseal thickness	Sham vs OVX	105.94	0.005*
	OVX vs High Dose (P1)	−90.40	0.014*
	OVX vs Low Dose (P2)	−72.81	0.044*
	OVX vs Risedronat	−85.75	0.020*
ALP	Sham vs OVX	25.32	0.050*
	OVX vs High Dose (P1)	−22.04	0.085
	OVX vs Low Dose (P2)	−29.51	0.024*
	OVX vs Risedronat	−29.35	0.025*
Kadar Col1a1	Sham vs OVX	3.31	0.033*
	OVX vs High Dose (P1)	−3.97	0.012*
	OVX vs Low Dose (P2)	−4.54	0.005*
	OVX vs Risedronat	−5.49	0.001*
Kadar Osteocalcin	Sham vs OVX	1.02	0.014*
	OVX vs High Dose (P1)	−0.83	0.042*
	OVX vs Low Dose (P2)	−0.82	0.044*
	OVX vs Risedronat	−1.54	0.001*

**p* significant if *p* < 0.05

Mechanism of MSC exosomes on osteoblast activity

The significant extent of bone regeneration noted in this study can be attributed to both soluble protein growth factors and the transmission of regulatory genetic information, specifically microRNAs (miRNAs), as components of the extensive paracrine signaling mechanisms employed by mesenchymal stem cells (MSCs) in regenerative applications [21,22]. This explanation corresponds with the accepted perspective that MSC-based therapies may operate via paracrine processes, rather than solely through direct cell replacement, encompassing cytoprotective and anti-apoptotic effects, as well as the control of tissue remodeling pathways [22]. Simultaneously, miRNAs are acknowledged as crucial regulators of osteogenic differentiation and bone regeneration due to their modulation of gene expression programs that dictate

lineage commitment and maturation [23]. This regulatory strategy corresponds with epigenetic control, characterized by modified gene expression without alterations in the DNA sequence, which is increasingly acknowledged as a mechanism affecting cell fate and tissue remodeling results [24].

This mechanistic framing is particularly relevant to bone-loss settings linked to sex-steroid deficiency and oxidative stress—2 nonheritable contributors to primary osteoporosis that can compromise bone-forming cell viability and function [25]. Because oxidative stress is implicated as a damaging cofactor in osteoporosis risk, any MSC-mediated paracrine mechanism that improves survival and osteogenic activity would be expected to contribute materially to the recovery of osteoblast populations and restoration of bone formation capacity [25]. Accordingly, the marker profile described (osteoblast number; early differentiation marker ALP;

matrix/mineralization markers such as Col1a1 and osteocalcin) can be organized into 3 testable molecular mechanisms in which miRNA-linked regulation converges on canonical osteogenic signaling—most prominently, the Wnt/ β -catenin axis and its downstream osteogenic transcriptional program [26,27].

The significant increase in osteoblast number described for the treatment group is interpreted as the outcome of (i) increased proliferation and/or (ii) decreased cell loss (e.g., reduced apoptosis), because net osteoblast abundance is determined by the balance of these processes in the local bone microenvironment [21]. In estrogen-deficiency-linked bone fragility, increased oxidative stress is a recognized risk factor that can worsen skeletal integrity and increase the vulnerability of bone-forming cells to damage, thereby diminishing osteoblast performance and persistence [25]. In this context, MSCs have been described as capable of exerting anti-apoptotic and protective effects via paracrine signaling, indicating that MSC-derived secreted signals can shift the survival balance in stressed tissues [21,22]. Therefore, a recovery of osteoblast numbers is consistent with an MSC-driven paracrine mechanism that enhances survival and/or restores proliferative competence in osteogenic compartments under oxidative stress-related conditions [25]. Within this model, miR-21 is proposed as a candidate miRNA that could contribute to the observed osteoblast rebound by biasing recipient cells toward growth/survival phenotypes (i.e., “reprogramming” at the post-transcriptional level), an idea that aligns with the general role of miRNAs as regulators of osteogenic differentiation programs and tissue regeneration [23]. Although the specific miR-21→target-gene mappings stated (e.g., *Spry2* repression) are not directly documented within the provided references, the logic that miRNA-mediated gene regulation can alter osteogenic outcomes is supported by direct evidence that particular miRNAs can promote osteogenic differentiation and regeneration through repression of inhibitory nodes (e.g., repression of a Wnt antagonist by miR-129-5p enhances osteogenic differentiation and bone regeneration) [23]. Thus, the increase in osteoblast numbers can be treated as compatible with a miRNA-linked paracrine mechanism that functionally counteracts stress-associated bone loss and supports the rebuilding of bone-forming cell pools [21,23,25].

The simultaneous elevation of extracellular matrix and mineralization-associated markers (as represented by Col1a1 and osteocalcin) is consistent with enhanced osteoblast maturation and bone-forming activity, because osteogenic differentiation studies commonly track early markers (e.g., ALP) alongside later matrix/mineralization outputs to infer progression from commitment to functional bone formation [28,29]. In MSC osteogenic biology, differentiation is regulated by multi-pathway signaling and transcriptional control (including Wnt/ β -catenin and osteogenic transcription factors), which together coordinate the expression of bone formation genes and remodeling mediators [26,27,30]. Therefore, an increase in matrix/mineralization markers can be interpreted as the downstream consequence of strengthened osteogenic programming and maturation in the treated group, consistent with known frameworks of bone formation and remodeling control [26,30]. In this model, the miR-29 family (including miR-29b) is proposed as a mechanistic contributor to the matrix/mineralization phenotype. The relevance of the miR-29 family to bone turnover and canonical Wnt-pathway-linked marker patterns is supported by clinical/translational evidence connecting miR-29a to the expression of bone turnover markers within the canonical Wnt signaling context [31]. Because canonical Wnt signaling is strongly implicated in osteoblastogenesis and bone mass regulation—promoting osteoblast activity and increasing bone mass—an miRNA family linked to Wnt-pathway marker patterns provides a biologically coherent bridge between miRNA regulation and enhanced matrix/mineralization outputs [26,31]. More broadly, the principle that miRNAs can act as upstream regulators of osteogenic differentiation and bone regeneration is directly supported by experimental evidence showing that miRNA-mediated repression of an inhibitory Wnt component (*Dkk3*) promotes osteogenesis and regeneration [23]. Hence, the elevated Col1a1/osteocalcin profile can be framed as consistent with miRNA-associated amplification of osteoblast maturation pathways that culminate in increased matrix formation and mineralization capacity [23,26,30].

The increase in alkaline phosphatase (ALP) is a hallmark compatible with enhanced early osteogenic differentiation, since ALP is routinely used as an osteogenic marker and is experimentally observed to

change with shifts in osteogenic programs and upstream regulatory signals in MSC-lineage cells [28]. Canonical Wnt/ β -catenin signaling is repeatedly identified as a central pro-osteogenic pathway: it promotes osteoblastogenesis, increases osteoblast activity, and supports increases in bone mass, while coupling signaling to osteogenic transcriptional outputs [26]. Mechanistically, Wnt/ β -catenin signaling is linked to RUNX2, which is described as a target associated with β -catenin/TCF nuclear signaling and is required for osteoblastic differentiation and bone formation [27]. Complementarily, regulation of β -catenin stability (e.g., via GSK3 β activity) is central to Wnt- β -catenin pathway output and has been explicitly connected to new bone formation contexts, underscoring that stronger β -catenin signaling can intensify osteogenic differentiation cascades [32]. A critical control point in this axis is the existence of extracellular or intracellular Wnt antagonists. For example, DKK1 is a canonical Wnt/ β -catenin pathway inhibitor; experimental Wnt inhibition via DKK1 prevents β -catenin signaling, demonstrating that DKK-family antagonists can suppress canonical Wnt outputs [33]. Moreover, DKK1 and soluble frizzled-related proteins (sFRPs) are consistently expressed in stromal fibroblast populations within inflammatory joint disease contexts, underscoring that these antagonists function as physiologically pertinent inhibitors of Wnt signaling in musculoskeletal tissue [34]. The significance of balanced Wnt activity is underscored by studies indicating that excessive or dysregulated canonical Wnt activation can be detrimental to cartilage, suggesting that Wnt signaling must be modulated with proper magnitude and timing to preserve tissue homeostasis [35]. Taken together, these findings support a mechanistic structure in which reducing effective Wnt antagonism can increase β -catenin signaling and thereby enhance RUNX2-linked osteogenic differentiation outputs, including early markers such as ALP [26-28,33].

In this model, miR-196a and let-7 family miRNAs are proposed as candidate regulators that could contribute to the ALP increase by shifting the Wnt/ β -catenin signaling balance toward activation (e.g., by reducing Wnt-antagonist influence). While the provided references do not directly document miR-196a/let-7 targeting of specific Wnt inhibitors, the underlying mechanistic premise—that miRNAs can promote

osteogenesis by repressing Wnt antagonists—is directly demonstrated by the finding that miR-129-5p promotes osteogenic differentiation and bone regeneration via repression of Dkk3 (a Wnt antagonist) [23]. Because Wnt activation is upstream of β -catenin nuclear signaling and RUNX2-dependent osteogenic differentiation [26,27,32], and because ALP is a measurable readout of early osteogenic differentiation state [28], the observed ALP increase is consistent with a miRNA-linked paracrine mechanism that functionally disinhibits canonical Wnt signaling during early osteogenic commitment [23,26-28].

When considered together, the reported pattern—(i) increased osteoblast number, (ii) increased ALP, and (iii) increased matrix/mineralization markers—aligns with a stepwise strengthening of osteogenesis from early commitment (ALP) to lineage-defining transcriptional programming (RUNX2) and ultimately functional bone formation (matrix production/mineralization), which is consistent with established osteogenic differentiation logic and MSC osteogenesis readouts [27-29,36]. Canonical Wnt/ β -catenin signaling provides a unifying pathway that can explain coordinated increases across these stages, given its documented roles in promoting osteoblastogenesis and bone mass and its mechanistic linkage to RUNX2 [26,27]. At the same time, the stress-related osteoporosis background (sex-steroid deficiency and oxidative stress) provides a plausible reason why protective paracrine actions that reduce apoptosis or improve resilience would meaningfully amplify recovery of osteoblast populations and downstream osteogenesis [21,25].

Thus, although direct evidence for the specific miR-21/miR-29b/miR-196a/let-7 target interactions named in the task is not contained within the provided reference set, the overall hypothesis that miRNA-linked regulation can drive substantial osteogenic recovery—particularly via disinhibition or activation of canonical Wnt/ β -catenin signaling and reinforcement of RUNX2-dependent osteoblast differentiation—is supported by convergent evidence on (i) Wnt/ β -catenin–RUNX2 control of osteogenesis [26,27,32], (ii) inhibitory roles of DKK-family antagonists in suppressing β -catenin signaling [33,34], and (iii) direct demonstration that an miRNA can promote osteogenesis and regeneration by repressing a Wnt antagonist (Dkk3) [23].

Finally, the fact that a peripheral/systemic route of administration produced therapeutic effects at a distant skeletal site is consistent with the general principle that EVs are studied as transferable, circulating bioactive carriers capable of delivering functional cargo to recipient cells, which underpins their development as therapeutic and delivery platforms [37]. Because MSCs and stem-cell-based interventions can act through immunomodulatory and paracrine mechanisms rather than solely via local engraftment, systemic delivery of MSC-EVs provides a plausible route by which osteogenic and immunoregulatory signals could influence bone remodeling at target sites beyond the injection location [38]. More broadly, given that inflammatory and tissue-stress contexts are associated with altered Wnt/ β -catenin signaling and osteogenic marker regulation, systemically distributed EV cargo that impacts these pathways remains a coherent mechanistic explanation for distant-site osteogenic effects observed after administration [39].

Despite these promising findings, several limitations of this study must be acknowledged. First, although histomorphometric analysis confirmed a significant increase in trabecular thickness, we did not perform biomechanical testing (e.g., 3-point bending or compression tests) to quantify functional bone strength directly. Although structural improvement typically correlates with mechanical integrity, direct biomechanical validation remains a gold standard for future investigations. Second, the proposed molecular mechanisms involving specific miRNAs (e.g., miR-21, miR-29b) were inferred from established literature regarding MSC-derived exosome cargo. We did not conduct RNA sequencing or specific miRNA profiling on the exosome batches used in this study to confirm the abundance of these transcripts. Third, a notable limitation of the present study is the significant difference in baseline Alkaline Phosphatase (ALP) levels among the experimental groups ($p = 0.010$). This initial physiological variability, likely resulting from individual biological responses during the 6-week ovariectomy-induced bone loss period, could potentially introduce bias if final outcomes were compared in isolation. To rigorously mitigate this issue and ensure an accurate assessment of the treatment's true effect, we employed paired t-tests to evaluate the within-group magnitude of change (delta change) from baseline to the

end of the intervention. By focusing on the longitudinal change within each subject rather than absolute post-treatment values, we were able to minimize the confounding effect of baseline disparities and isolate the specific anabolic impact of the MSC-derived exosomes. Future research should address these gaps by incorporating biomechanical strength assessments and comprehensive cargo profiling to fully elucidate the structure-function relationship and the precise molecular drivers of exosome-mediated bone repair.

Conclusions

This study concluded that the administration of Mesenchymal Stem Cell (MSC) exosomes was shown to be effective in restoring osteoblast activity and improving bone microarchitecture in a mouse model of post-menopausal osteoporosis. Specifically, exosome therapy can significantly increase the number of osteoblasts and bone metaphyseal thickness, as well as restore the expression of key osteogenic markers (ALP, Colla1, and Osteocalcin) to near-normal physiological levels. Another important finding is that the regenerative efficacy of MSC exosomes, especially at high doses, is statistically equivalent to that of standard therapy, Risedronate. This indicates that MSC exosomes not only work through a protective (preservative) mechanism but also have strong anabolic potential. Therefore, MSC exosomes have great potential to be further developed as a safe and effective cell-free therapy strategy for the treatment of osteoporosis.

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Declaration of generative AI in scientific writing

Grammarly was employed exclusively for grammar correction and refinement of language expression. Gemini is used to create illustrative images for research design and abstracts. The conception of the study, literature synthesis, data analysis, interpretation of findings, and all aspects of critical discussion were entirely conducted by the author without reliance on AI.

CRedit author statement

Nurhasan Agung Prabowo: Conceptualization, Methodology, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Visualization, Project administration. **Ida Nurwati:** Methodology, Validation, Writing - Review & Editing, Supervision. **Tonang Dwi Ardyanto:** Validation, Writing - Review & Editing, Supervision. **Eti Poncorini Pamungkasari:** Validation, Writing - Review & Editing. **Arief Nurudhin:** Validation, Writing - Review & Editing. **Brian Wasita:** Investigation, Writing - Review & Editing. **Paramasari Dirgahayu:** Conceptualization, Methodology, Resources, Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

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