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Dose-Dependent Effects of ZnO Nanoparticles on the Osteogenic Differentiation Potential of Rat-Derived MSCs

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ABSTRACT

Zinc oxide nanoparticles (ZnO NPs) have shown promise in bone tissue engineering applications due to their osteogenic properties. The purpose of this research was to analyze how varying doses of ZnO NPs modulate the in vitro rats bone marrow-sourced mesenchymal stem cells (MSCs) undergoing osteogenic conversion (OD). Sol-gel technique was employed to synthesize ZnO NPs, which were then analyzed using FTIR, XRD, and TEM. MSCs obtained from rat bone marrow were maintained in osteogenic medium containing ZnO NPs at 0, 10, 25, and 50 µg/mL concentrations. Assessment of cell viability was carried out using the MTT assay. OD was assessed by analyzing osteogenic marker gene expression (Runx2, OSX, and OCN), calcium deposition, and the alkaline phosphatase (ALP) activity, using real-time PCR. ZnO NPs were successfully synthesized and characterized, exhibiting a rod-shaped morphology with an average length of 50 nm. ZnO NPs at concentrations up to 25 µg/mL did not significantly affect cell viability. Calcium deposition and ALP activity were significantly enhanced in MSCs exposed to ZnO NPs at 10 and 25 µg/mL concentrations relative to the control. The expression of OCN, OSX, and Runx2 was significantly upregulated in a manner that is dependent on the dosage in ZnO NP-treated MSCs, with the highest expression levels observed at 25 µg/mL. ZnO NPs at concentrations up to 25 µg/mL enhance the OD of rat bone marrow-derived MSCs in vitro, as demonstrated by the escalation in calcium deposition, ALP activity, and upregulation of osteogenic marker genes. These findings indicate that ZnO NPs could serve as a promising bioactive agent in bone tissue engineering scaffolds to promote bone regeneration. To better understand the fundamental mechanisms and assess the in vivo performance of scaffolds containing ZnO NPs, further investigations are necessary.

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INTRODUCTION

Bone tissue engineering has presented itself as an auspicious avenue to address the growing need for bone regeneration and repair, particularly in cases of large bone defects, non-union fractures, and osteoporosis [1,2]. The development of novel biomaterials that can stimulate the osteogenic differentiation (OD) of mesenchymal stem cells (MSCs) has been a key focus in this field [3]. Among the various nanomaterials explored for bone tissue engineering applications, the unique characteristics and bone growth enhancement potential of ZnO NPs, also known as zinc oxide nanoparticles, have garnered a lot of interest [4].

Zinc is an essential trace element which performs an essential part in bone metabolism and mineralization [5]. Research has revealed that it promotes collagen synthesis, stimulates osteoblast differentiation, and inhibits osteoclast activity [6]. Moreover, zinc deficiency has been associated with impaired bone growth and increased risk of osteoporosis [7]. The incorporation of zinc into biomaterials has been explored as a strategy to improve their osteogenic properties [8]. However, the use of ZnO NPs has gained particular interest due to their high surface area to volume ratio, which allows for enhanced interaction with biological systems [9,10].

Several studies have investigated the effects of ZnO NPs on the OD of MSCs. For instance, Khader and Arinzeh [11] reported that ZnO NPs promoted the OD of human adipose-derived MSCs, as demonstrated by the escalation in calcium deposition and alkaline phosphatase (ALP) activity. Similarly, Bozorgi et al. [12] found that ZnO NPs enhanced the OD of rat bone marrow-derived MSCs, with upregulation of osteogenic marker genes like Runx2 and osteocalcin (OCN). However, the optimal concentration of ZnO NPs for promoting OD and the root causes are still to be thoroughly explained.

The physicochemical properties of ZnO NPs, like size, shape, and surface chemistry, can significantly influence their biological effects [13]. Therefore, it is crucial to characterize the synthesized ZnO NPs thoroughly to understand their interaction with MSCs. Various synthesis methods have been employed to produce ZnO NPs, including sol-gel, hydrothermal, and precipitation techniques [14]. The sol-gel method has been widely used due to its simplicity, low cost, and ability to control the particle size and morphology [15].

In addition to their osteogenic properties, ZnO NPs have also been shown to exhibit antimicrobial activity [16]. This is particularly relevant in the context of bone tissue engineering, as implant-associated infections are a major challenge that can lead to implant failure and revision surgeries [17]. The incorporation of ZnO NPs into bone tissue engineering scaffolds could potentially provide a dual benefit of promoting bone regeneration and preventing bacterial colonization [18].

Despite the promising results reported in the literature, there are still several challenges and limitations associated with bone tissue engineering regarding the application of ZnO NPs. It could potentially be cytotoxic at a specific concentration which is of concern [19]. It is essential to determine the safe and effective concentration range of ZnO NPs that can promote OD without causing adverse effects on cell viability. Another challenge is the long-term fate and biodegradation of ZnO NPs in vivo, which requires further investigation [20,21].

The current study aims to address some of these challenges and provide an extensive understanding of the dose-dependent impacts of ZnO NPs on the OD of rat bone marrow-derived MSCs. We hypothesized that ZnO NPs would enhance OD in a dose-dependent manner up to a certain concentration, beyond which cytotoxic effects may occur. To test this hypothesis, we synthesized ZnO NPs using a sol-gel method and characterized their physicochemical properties using X-ray diffraction (XRD), Fourier-transform infrared spectroscopy (FTIR), and transmission electron microscopy (TEM). MSCs were isolated from rat bone marrow and cultured in osteogenic medium enriched with ZnO NPs in various amounts (0, 10, 25, and 50 μg/mL). The effects of ZnO NPs on cell viability, OCN, OSX (osterix), and Runx2 gene expression, along with calcium deposition and ALP activity were evaluated.

MATERIALS AND METHODS

Type of Study and Method

This study employed an in vitro experimental design to analyze the dose-dependent effects of ZnO NPs on the OD of rat MSCs. The study utilized a quantitative approach, assessing various parameters such as cell viability, gene expression of osteogenic markers, calcium accumulation, and the activity of ALP. The experimental data were collected over a period of 21 days, with specific time points for various assays. Cell viability was

assessed at days 1, 3, and 7. The activity of ALP was gauged on the 7th and 14th days, while calcium deposition was evaluated at day 21. Gene expression analysis of osteogenic markers (Runx2, OSX, and OCN) was performed at days 7 and 14. The study was conducted at the Biomedical Engineering Laboratory, University of Baghdad. The laboratory is equipped with state-of-the-art facilities for nanomaterial synthesis, cell culture, and analytical techniques.

Male Wistar rats (weighing 185-240 g, 8-10 weeks old) made up the study population. A total of 12 rats were used for the isolation of bone marrow-derived MSCs. The sample size was determined based on previous studies and statistical power analysis, hinge on a 0.05 significance level and a 0.8 power. The experimental procedures were carried out in a biosafety level 2 (BSL-2) laboratory, following standard guidelines for cell culture and nanomaterial handling. A constant temperature of 37°C, 95% humidity, and a 5% CO2 atmosphere were maintained in the laboratory.

Data Collection Tool

ZnO NP synthesis and characterization

ZnO NPs were synthesized utilizing a sol-gel technique as explained by Shafiee et al. [22] with slight modifications. In summary, zinc acetate dihydrate was dissolved in ethanol under stirring at 60°C. Under vigorous stirring, sodium hydroxide solution was introduced gradually to the zinc acetate solution until a white precipitate formed. After centrifugation, the precipitate was washed with ethanol and deionized water, then dried overnight at 80°C. For 2 hours, the acquired ZnO NPs were calcined at 500°C.

By employing TEM, XRD, and FTIR, the synthesized ZnO NPs were characterized. TEM was used to observe the morphology and size of the ZnO NPs. To confirm the crystalline structure of the ZnO NPs, XRD has been utilized. FTIR has been applied to identify the surface functional groups of the ZnO NPs.

MSC isolation and culture

The procedure explained by Huang et al. [23] was employed to isolate rat bone marrow-derived MSCs. Briefly, the femurs and tibias were dissected from the rats, and using a syringe loaded with a solution of DMEM (Dulbecco's Modified Eagle's Medium), the bone marrow was flushed out. The bone marrow suspension was centrifuged, and the team employed DMEM supplemented with 10% FBS (fetal bovine serum) and 1% penicillin-streptomycin to resuspend the cell pellet. At 37°C, incubation of the cells was carried out with 5% CO2 after seeding them in tissue culture flasks. After 48 hours, the non-adherent cells were removed and the medium was replaced at 3-day intervals. For the following experiments, the researchers utilized MSCs between passages 3 and 5.

OD and ZnO NP treatment

MSCs were introduced into 24-well plates at a seeding density of 5 × 104 cells in each well and cultured in osteogenic medium (DMEM supplemented with 80 nM dexamethasone, 45 µM ascorbic acid, 8 mM β-glycerophosphate, and10% FBS) [24]. ZnO NPs were applied to the osteogenic medium at concentrations of 0 (control), 10, 25, and 50 µg/mL. Every three days, the medium underwent a change.

Data Collection Method

Cell viability assay

The MTT assay determined cell viability [25]. At a density of 1 × 104 cells per well, MSCs were seeded in 96-well plates and administered with ZnO NPs at varying concentrations for 1, 3, and 7 days. MTT solution (5 mg/mL) was introduced into each well and maintained for 4 hours at 37°C. Using dimethyl sulfoxide (DMSO), the formazan crystals were dissolved followed by absorbance measurement at 570 nm utilizing a BioTek ELx800 microplate reader.

ALP activity assay

A colorimetric assay kit (Abcam, ab83369) was employed to evaluate ALP activity. MSCs were treated with ZnO NPs for 7 and 14 days. The cells were lysed with assay buffer, and the p-nitrophenyl phosphate (pNPP), the substrate, was incubated with the lysate for 60 minutes at 25°C. Following the termination of the reaction with stop solution, the absorbance was measured at 405 nm using a microplate reader.

Calcium deposition assay

Alizarin Red S (ARS) staining was employed to evaluate calcium deposition [24]. MSCs were treated with ZnO NPs for three weeks. The cells were stained with 2% ARS solution (pH 4.2), fixed with 4% paraformaldehyde for 20 minutes, and washed with distilled water. The stained

Table 1. Primer sequences used for real-time PCR.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
Runx2	CGCATTCCTCATCCCAGTAT	GCCTGGGGTCTGTAATCTGA
OSX	AGGCACAAAGAAGCCATAC	AATGAGTGAGGGAAGGGT
OCN	CTGACCTCACAGATCCCAAGC	TGGTCTGATAGCTCGTCACAAG
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA

calcium deposits have been dissolved in 10% cetylpyridinium chloride (CPC), and a microplate reader was used to quantify the absorbance at 562 nm.

xpression analysis

Total RNA was isolated from MSCs treated with ZnO NPs for 7 and 14 days using TRIzol reagent (Invitrogen). cDNA was synthesized from RNA using a High-Capacity cDNA Reverse Transcription Kit supplied by Applied Biosystems. An Applied Biosystems StepOnePlus Real-Time PCR System was employed to perform real-time PCR using SYBR Green PCR Master Mix (Applied Biosystems). The primers used for Runx2, OSX, OCN, and GAPDH (internal control) are listed in Table 1. The 2-ΔΔCt method was used to calculate the relative gene expression [26].

Data Analysis Method

The results, calculated as mean ± standard deviation (SD), were based on data from at least three separate experiments. Multiple comparisons were made employing a one-way ANOVA followed by Tukey's post hoc test. Statistical significance was assigned to p-values below 0.05.

RESULTS AND DISCUSSION

ZnO NP Characterization

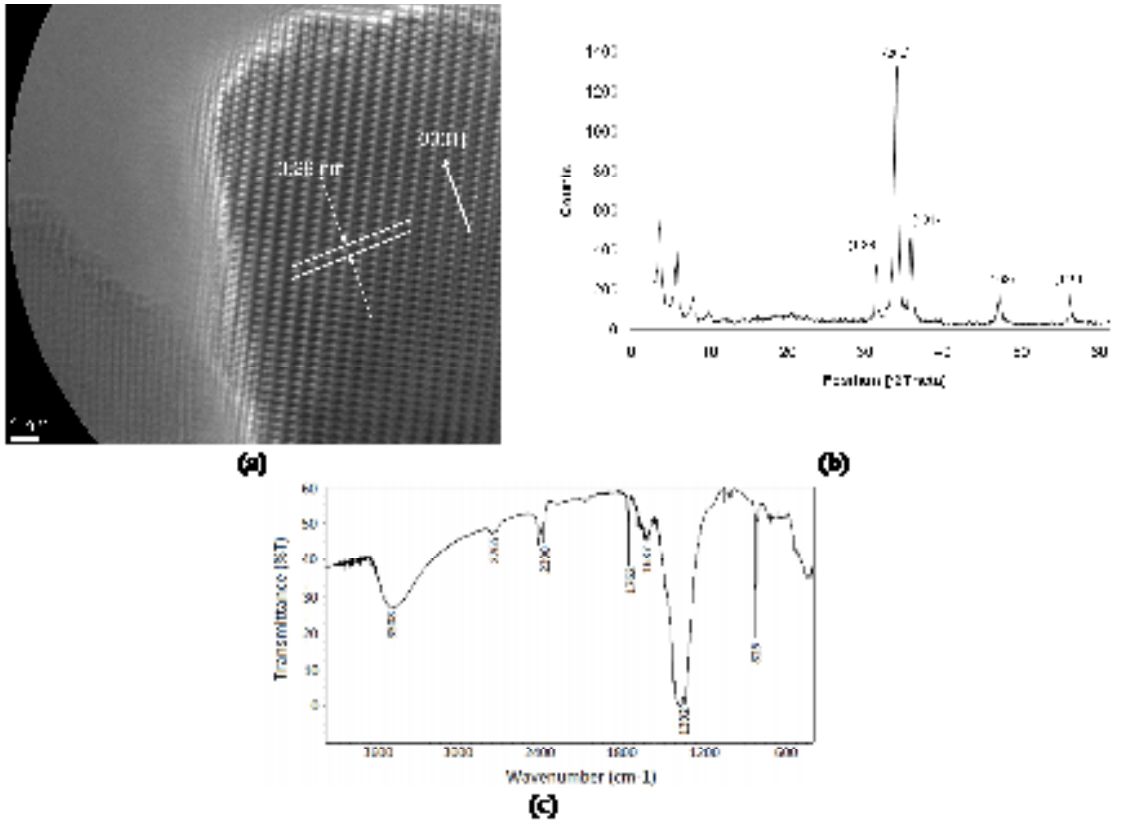

Fig. 1. Characterization of ZnO NPs synthesized using the sol-gel method: (a) TEM images showing the rod-shaped morphology and size of the ZnO NP; (b) XRD pattern confirming the hexagonal wurtzite crystal structure of the ZnO NPs; (c) FTIR spectrum demonstrating the presence of the characteristic Zn-O bond.

The synthesized ZnO NPs were characterized using TEM, XRD, and FTIR. TEM images (Fig. 1a) revealed that the ZnO NPs were rod-shaped with an average length of 50 ± 10 nm and a diameter of 10 ± 2 nm. The XRD pattern (Fig. 1b) exhibited sharp peaks at 2θ values of 68.0°, 62.9°, 56.6°, 47.6°, 36.3°, 34.5°, and 31.8°, corresponding to the (112), (103), (110), (102), (101), (002), and (100) planes, respectively, of the hexagonal wurtzite structure of ZnO. The FTIR spectrum (Fig. 1c) showed a strong absorption band at 437 cm-1, attributed to the stretching vibration of the Zn-O bond. These results confirm the successful synthesis of crystalline ZnO NPs with a rod-like morphology.

Cell Viability

The MTT assay was used to study the effect of ZnO NPs on MSC viability. As shown in Table 2, cell viability was not significantly affected by ZnO NPs at 10 and 25 µg/mL concentrations compared to the control at all time points ($p > 0.05$). At a concentration of 50 µg/mL, a significant decrease in cell viability was noted on days 3 and 7 ($p < 0.05$). These findings indicate that ZnO NPs at concentrations up to 25 µg/mL do not exert cytotoxic effects on MSCs.

ALP Activity

ALP activity, an early marker of OD, was assessed in MSCs treated with ZnO NPs for 7 and 14 days. As presented in Table 3, ALP activity was significantly increased in MSCs treated with ZnO NPs at all concentrations relative to the control at both time points ($p < 0.05$). The maximum ALP activity has been detected in MSCs treated with 25 µg/mL ZnO NPs, with a 2.8-fold increase at day 7 and a 3.5-fold increase at day 14 compared to the control ($p < 0.01$). These findings indicate that ZnO NPs enhance the OD of MSCs in a dose-dependent manner.

Calcium Deposition

The effect of ZnO NPs on calcium deposition, a late marker of OD, was evaluated using ARS staining after 21 days of treatment. Table 4 demonstrates that ZnO NPs significantly increased calcium deposition in MSCs at all concentrations relative to the control ($p < 0.05$). The maximum calcium deposition was observed in MSCs treated with 25 µg/mL ZnO NPs, with a 4.2-fold increase relative to the control ($p < 0.001$). The aforementioned findings further confirm the osteogenic potential

Table 2. Cell viability of MSCs treated with ZnO NPs at different concentrations and time points.

ZnO NP Concentration (µg/mL)	Cell Viability (% of control)		
	Day 1	Day 3	Day 7
0 (Control)	100 ± 5	100 ± 6	100 ± 4
10	98 ± 4	95 ± 5	94 ± 3
25	97 ± 3	93 ± 4	92 ± 5
50	95 ± 6	80 ± 7*	80 ± 8*

Note: *p < 0.05 compared to the control.

Table 3. ALP activity in MSCs treated with ZnO NPs at different concentrations and time points.

ZnO NP Concentration (µg/mL)	ALP Activity (U/mg protein)	
	Day 7	Day 14
0 (Control)	0.5 ± 0.1	0.8 ± 0.2
10	0.9 ± 0.2*	1.8 ± 0.3*
25	1.4 ± 0.3**	2.8 ± 0.4**
50	1.1 ± 0.2*	2.2 ± 0.3*

Note: *p < 0.05, **p < 0.01 compared to the control.

Table 4. Calcium deposition in MSCs treated with ZnO NPs at different concentrations on day 21.

ZnO NP Concentration (µg/mL)	Calcium Deposition (mM/well)
0 (Control)	1.2 ± 0.3
10	3.0 ± 0.5*
25	5.0 ± 0.8***
50	3.7 ± 0.6**

Note: *p < 0.05, **p < 0.01, ***p < 0.001 compared to the control

of ZnO NPs and their ability to promote matrix mineralization in MSCs.

Gene Expression Analysis

The osteogenic marker genes expression (Runx2, OSX, and OCN) in MSCs treated with ZnO NPs for 7 and 14 days was analyzed using real-time PCR. According to Table 5, there was a significant upregulation in the expression of all three genes in MSCs treated with ZnO NPs in comparison to the control at both time points ($p < 0.05$). The highest expression levels were observed in MSCs treated with 25 µg/mL ZnO NPs, with fold increases of 3.2, 4.1, and 4.6 for Runx2, OSX, and OCN, respectively, at day 14 relative to the control ($p < 0.001$). The aforementioned findings demonstrate that ZnO NPs enhance the expression of key transcription factors and proteins included in OD in a manner dependent on the dose.

Table 6 summarizes the main findings of the study, presenting the fold changes in calcium deposition, ALP activity, and gene expression in MSCs treated with varying concentrations of ZnO NPs relative to the control.

The results presented in Table 6 demonstrate that ZnO NPs enhanced the OD of MSCs in a dose-responsive way, with the most pronounced effects occurring at a concentration of 25 µg/mL. Calcium deposition, ALP activity, and the expression of osteogenic marker genes were significantly increased in MSCs treated with ZnO NPs compared to the control, indicating their potential to promote bone formation.

This research analyzed the dose-dependent effects of ZnO NPs on the OD of rat bone marrow-derived MSCs. The main findings of this study demonstrate that ZnO NPs, at concentrations up to 25 µg/mL, significantly enhanced calcium deposition, ALP activity, and the osteogenic marker genes expression (OCN, OSX, and Runx2) compared to the control, without exhibiting cytotoxic effects. These results suggest that ZnO NPs have the potential to be used as a bioactive agent in bone tissue engineering scaffolds to promote bone regeneration.

The osteogenic properties of ZnO NPs have been reported in previous studies. For instance, Raghav et al. [27] found that ZnO NPs promoted the OD of human adipose-derived MSCs, as demonstrated by the escalation in calcium deposition and ALP activity. Similarly, Li et al. [28] reported that ZnO NPs enhanced the OD of rat bone marrow-derived MSCs, with upregulation of osteogenic marker genes like OCN and Runx2. The evidence gathered in this research implies consistency with these reports, further confirming the osteogenic potential of ZnO NPs.

Still, the optimal concentration of ZnO NPs for promoting OD has been a subject of debate. The present research revealed that a 25 µg/mL ZnO NPs concentration had the most pronounced effects on OD, while higher concentrations (50 µg/mL) led to reduced cell viability. These findings correspond to the discovery of Bosch-Rué et al. [8], who reported that ZnO NPs at a concentration of 20 µg/mL significantly enhanced the OD of

Table 5. Relative gene expression of osteogenic markers in MSCs treated with ZnO NPs at different concentrations and time points.

ZnO NP Concentration (µg/mL)	Relative Gene Expression (Fold Change)					
	Runx2		OSX		OCN	
	Day 7	Day 14	Day 7	Day 14	Day 7	Day 14
0 (Control)	1.0±0.1	1.0±0.2	1.0±0.2	1.0±0.1	1.0±0.2	1.0±0.1
10	1.5±0.3*	2.1±0.4*	1.8±0.4*	2.5±0.5**	2.0±0.3*	2.9±0.5*
25	2.3±0.5***	3.2±0.6****	2.7±0.6***	4.1±0.8****	3.1±0.7**	4.6±0.9****
50	1.8±0.4*	2.4±0.5*	2.1±0.5*	3.0±0.6***	2.4±0.5*	3.4±0.7***

Note: *p < 0.05, **p < 0.01, ***p < 0.001 compared to the control.

Table 6. Fold changes in osteogenic markers in MSCs treated with ZnO NPs compared to the control.

ZnO NP Concentration (µg/mL)	ALP Activity		Calcium Deposition	Gene Expression (Day 14)		
	Day 7	Day 14	Day 21	Runx2	OSX	OCN
10	1.8*	2.3*	2.5*	2.1*	2.6*	2.5*
25	2.8***	3.5***	4.2****	3.2****	4.1****	4.6****
50	2.1**	2.7**	3.1***	2.4*	3.0***	3.4***

Note: *p < 0.05, **p < 0.01, ***p < 0.001 compared to the control.

human periodontal ligament stem cells (PDLSCs), while higher concentrations (50 and 100 µg/mL) induced cytotoxicity. In contrast, Rossner Jr et al. [29] found that ZnO NPs at a concentration of 50 µg/mL promoted the OD of mouse MSCs without causing cytotoxicity. These discrepancies may be attributed to differences in cell types, ZnO NP characteristics, and experimental conditions.

The mechanisms underlying the osteogenic effects of ZnO NPs have been explored in several studies. It has been suggested that ZnO NPs can promote OD by activating the Wnt/β-catenin signaling pathway [10], which is fundamental in regulating bone formation and remodeling. Additionally, ZnO NPs have been shown to upregulate BMP-2 (bone morphogenetic protein-2) expression [20], a potent osteogenic growth factor that induces the differentiation of MSCs into osteoblasts. Further research is needed to elucidate the specific molecular mechanisms by which ZnO NPs enhance OD in our study.

The incorporation of ZnO NPs into bone tissue engineering scaffolds has shown promising results in terms of improving their mechanical properties and bioactivity. For instance, Wang et al. [30] developed a ZnO NP-incorporated hydroxyapatite scaffold that exhibited enhanced compressive strength and promoted the OD of human MSCs. Similarly, Ghosh et al. [31] fabricated a ZnO NP-functionalized chitosan scaffold that displayed improved mechanical properties and stimulated the OD of rat MSCs. Based on the findings of our study, we suggest that incorporating ZnO NPs at a concentration of 25 µg/mL into bone tissue engineering scaffolds could potentially enhance their osteoinductive properties and promote bone regeneration.

One of the limitations of the present study is that it was conducted in vitro using rat bone marrow-derived MSCs. While these cells are widely used as a model for OD, the results may not fully translate to human MSCs or in vivo conditions. Future studies should investigate the effects of ZnO NPs on human MSCs and assess their efficacy in animal models of bone defects. Additionally, the long-term fate and biodegradation of ZnO NPs in vivo need to be thoroughly evaluated to ensure their safety and biocompatibility.

CONCLUSION

This study demonstrates the dose-dependent effects exerted by ZnO NPs on the OD of rat bone marrow-derived MSCs. ZnO NPs were successfully synthesized using the sol-gel method and characterized using TEM, XRD, and FTIR, confirming their rod-shaped morphology, hexagonal wurtzite crystal structure, and the presence of the characteristic Zn-O bond. The results showed that ZnO NPs at concentrations up to 25 µg/mL significantly enhanced the osteogenic differentiation of MSCs without causing cytotoxicity. This was evident from the increased ALP activity, calcium deposition, and upregulation of important osteogenic marker genes, like OCN, OSX, and Runx2. The most pronounced effects were seen at 25 µg/mL concentration, OCN gene expression, calcium deposition, and ALP activity showed fold increases of 4.6, 4.2, and 2.8, respectively, compared to the control.

The results indicate that ZnO NPs could potentially serve as a bioactive agent in bone tissue engineering scaffolds to facilitate the process of bone restoration. The incorporation of ZnO NPs at a 25 µg/mL optimal concentration could enhance the osteoinductive properties of scaffolds and stimulate bone formation. Still, more investigations are essential to clarify the basic molecular processes, assess the efficacy of ZnO NP-incorporated scaffolds in vivo, and evaluate their long-term safety and biocompatibility. The development of ZnO NP-based strategies for bone tissue engineering could potentially advance the treatment of bone defects and improve patient outcomes. This investigation lays the groundwork for subsequent studies on the application of ZnO NPs in regenerative medicine and highlights their promise as a novel biomaterial for bone tissue engineering.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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