RESEARCH PAPER

Biocompatibility And Osteogenic Assessment Of Copper Zinc Iron Oxide ($CuZnFe_2O_4$) Nanoparticles Intended For Bone Regeneration

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Abstract:

Introduction: Bone tissue engineering is emerging as a promising solution to address skeletal defects caused by trauma, disease, or aging. Conventional approaches such as autografts and allografts are limited by donor site morbidity, immune rejection, and availability. Nanoparticles, particularly magnetic nanoparticles like Copper Zinc Iron Oxide ($CuZnFe_2O_4$), are gaining attention due to their biocompatibility and potential to enhance osteogenic differentiation. Trace elements such as copper, zinc, and iron participate in bone metabolism, angiogenesis, and collagen synthesis, which makes $CuZnFe_2O_4$ nanoparticles an attractive material for bone regeneration. Thus, this study explores the biocompatibility and osteogenic potential of $CuZnFe_2O_4$ nanoparticles using mouse mesenchymal stem cells.

Materials and Methods: Mouse MSCs (mesenchymal stem cells) were cultured with $CuZnFe_2O_4$ nanoparticles at various concentrations of 5-50 µg/mL. The cytotoxicity of the nanoparticles was measured with the MTT assay of mitochondrial activity and the LDH assay of membrane integrity after 24 hours. The osteogenic differentiation of cells was determined by the quantitative PCR of the expression levels of Runx2, ALP, and Col-I under osteogenic conditions for seven days. The analysis was done using one-way ANOVA.

Results and Discussion: The $CuZnFe_2O_4$ nanoparticles were found to exhibit biocompatibility up to a concentration of 10 µg/mL, although minor cytotoxicity appeared in the MTT and LDH assays. Moreover, gene expression analysis showed elevated expression of Runx2, ALP, and Col-I under osteogenic conditions, thus indicating potential increased osteogenic differentiation capacity.

The concentrations of >20 µg/mL show cytotoxicity, requiring further optimization for concentrations.

Conclusion: $CuZnFe_2O_4$ nanoparticles show excellent biocompatibility and osteogenic potential as a promising biomaterial for bone tissue engineering. Further, *in vivo* studies and long-term biocompatibility should be carried out to assess their clinical applicability.

Keywords: Biocompatibility, Bone regeneration, Copper Zinc Iron Oxide nanoparticles, Mesenchymal stem cells, Osteogenic differentiation, Osteogenesis.

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Introduction:

Bone tissue engineering is an emerging field that promises to meet the increasing demand for efficient treatments of skeletal defects resulting from trauma, disease, or aging. Traditional methods, including autografts and allografts, have been widely used in clinical settings but are often associated with limitations, including donor site morbidity, immune rejection, and limited availability (G. Zhang et al. 2024) . This includes recent research attention towards the

application of nanoparticles in regenerative medicine, attributed to their unique physicochemical properties, biocompatibility, and ability to enhance cellular responses. Of these, magnetic nanoparticles, especially those based on metal oxides, have attracted special attention toward their application in promoting bone regeneration because of their ability to influence cellular behavior and facilitate osteogenic differentiation (Byun et al. 2023).

Copper Zinc Iron Oxide or $CuZnFe_2O_4$ nanoparticles are considered a class of magnetic nanoparticles that exhibit high stability and biocompatibility, along with multifunctionality. Copper, zinc, and iron are trace elements indispensable for many physiological processes, such as cellular metabolism, antioxidant defense, and remodeling of bones (Ling et al. 2025). Copper is involved in angiogenesis and collagen crosslinking, and zinc is an activator for many enzymes implicated in bone formation, including ALP. Iron is part of hemoglobin and other metalloproteins; it is critical for the transport of oxygen and cellular energy production (C.-Y. Wang et al. 2025). These elements in nanoparticles are expected to synergistically enhance osteoinductive potential while ensuring compatibility with living tissues.

The biocompatibility of the nanoparticles is crucial for their effective use in tissue engineering. Nanoparticles should not be cytotoxic and compromise the viability of the cells surrounding them (S. Zhang et al. 2024). MTT and LDH assays are widely used to study the cytotoxicity of nanoparticles by measuring cell viability and membrane integrity, respectively. These assays are important for understanding the safety profiles of nanoparticles and help determine concentration for therapeutic applications (Eldokmak et al. 2025).

However, in addition to the biocompatibility of these nanoparticles, their ability to induce osteogenic differentiation is critical for bone repair. Osteogenic differentiation is a cascade of molecular events that include the induction of transcription factors such as Runx2 and the upregulation of key osteogenic markers, including ALP and collagen type I, COL-I. These markers are crucial in matrix mineralization and bone formation (Ma et al. 2024). The osteoinductive property of nanoparticles can be gauged by observing the expression of these markers in cells that have been cultured under osteogenic conditions.

The aim of this study is to investigate the biocompatibility and osteogenic potential of $CuZnFe_2O_4$ nanoparticles as a candidate material in bone tissue engineering. Mouse mesenchymal stem cells (MSCs) were used as a model system to study the influence of these nanoparticles on cellular viability, membrane integrity, and osteogenic differentiation. The outcome of this study will offer invaluable information regarding the applicability of $CuZnFe_2O_4$ nanoparticles in bone regeneration processes and contribute to the fast-expanding knowledge on multifunctional nanoparticles for use in regenerative medicine. These nanoparticles have significant promise in addressing some of the present limitations of existing bone graft materials and push the advancement of the bone tissue engineering field by integrating biocompatibility with osteoinductive properties.

Materials and methods:

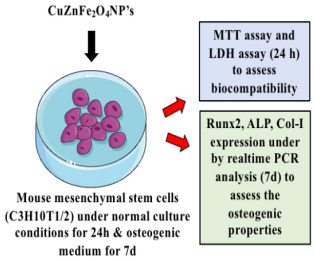

Figure 1: Schematic representation of the overall methodology adopted in the study. Mouse mesenchymal stem cells were treated the nanoparticles for 24h and 7d. MTT assay and LDH assay was performed to study the biocompatibility. Realtime PCR analysis was performed to study the influence of the nanoparticles on osteogenic markers.

Culture and Treatment: Mouse mesenchymal stem cells (MSCs) were treated with various concentrations of $CuZnFe_2O_4$ nanoparticles (5, 10, 20, and 50 ug/mL) for cytotoxicity and osteogenic differentiation studies. Treatment was done under standard culture conditions for 24 hours (for cytotoxicity assays) and up to 7 days (for gene expression analysis).

MSCs were cultured in α-MEM, supplemented with osteogenic inducers for differentiation studies. Media changes were performed every two days to maintain nutrient availability and nanoparticle exposure.

MTT Assay: Mitochondrial activity was quantified by measuring absorbance at 570 nm, indicating cell viability.MSCs were treated with nanoparticles for 24 hours.

MTT reagent (0.5 mg/mL) was added to each well, and cells were incubated for 4 hours at 37°C. The formazan crystals formed were dissolved in DMSO, and absorbance was measured at 570 nm using a microplate reader. Results were normalized to untreated controls to assess mitochondrial activity.

LDH Assay: LDH leakage into the conditioned media was measured as an indicator of membrane integrity and cytotoxicity.

LDH release was measured after 24 hours of nanoparticle treatment to assess membrane integrity. Conditioned media from treated cells were collected, and LDH activity was determined using a commercially available LDH assay kit according to the manufacturer's instructions. Absorbance was measured at 490 nm, and fold changes were calculated relative to untreated controls.

Gene Expression Analysis for Osteogenic Markers (Runx2, ALP, and Col-l): MSCs were cultured under osteogenic conditions for 7 days with or without $CuZnFe_2O_4$, nanoparticles (10ug/mL). qPCR was used to analyze the expression of osteogenic markers: Runx2, alkaline phosphatase (ALP), and collagen type I (Col-I).GAPDH served as the housekeeping gene.

MSCs were cultured in α-MEM supplemented with osteogenic inducers (50 ug/mL ascorbic acid, 10 mM B-glycerophosphate, and 100 nM dexamethasone) for up to 7 days in the presence or absence of $CuZnFe_2O_4$ nanoparticles (10 ug/mL). To analyze osteogenic differentiation, gene expression levels of key markers—Runx2, ALP, and collagen type I (Col-I)-were quantified using quantitative PCR (qPCR).

RNA concentration and purity were assessed, and CDNA synthesis was performed using a reverse transcription kit. qPCR was conducted using specific primers for Runx2, ALP, and Col-I. GAPDH was used as a housekeeping gene for normalization.

Statistical Analysis: All experiments were conducted in triplicates, and data were expressed as mean ± standard deviation (SD). Statistical significance was determined using one-way ANOVA.

Results:

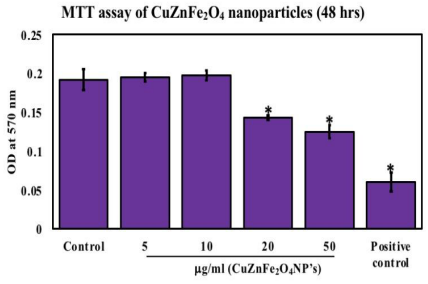

Figure 2: Biocompatibility assessment using MTT assay to analyze the non toxic dose of nanoparticles under normal culture conditions. Mouse mesenchymal stem cells were treated with NPs of varying concentrations for a period of 24h and MTT assay was performed. The results indicated the NP's exhibited significant toxicity beyond 10 µg/ml. The experiment was performed in triplicates (n=3). *- indicates significant increase compared to untreated control.

The control and low doses (5 ug/mL and 10 ug/mL) show no significant cytotoxicity, as indicated by stable OD values around 0.165-0.17. At higher concentrations (20 ug/mL and 50 mg/mL), significant toxicity is observed, evidenced by a marked decrease in OD values, indicating reduced cell viability. The positive control shows the lowest OD, representing high cytotoxicity. The nanoparticles are biocompatible at doses up to 10 ug/mL but exhibit significant toxicity at higher concentrations.

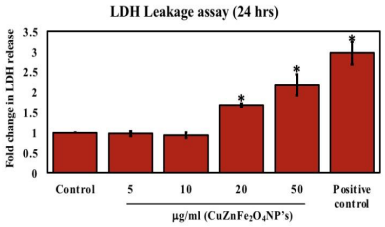

Figure 3: LDH release assay to analyze the non toxic dose of nanoparticles under normal culture conditions. Mouse mesenchymal stem cells were treated with NPs of varying concentrations for a period of 24h and LDH leakage assay was performed in the conditioned medium. The results indicated the NP's exhibited significant toxicity beyond 10 µg/ml. The experiment was performed in triplicates (n=3). *- indicates significant increase compared to untreated control.

Minimal LDH release is observed for the control and lower doses (5 g/mL and 10 ug/mL), indicating intact cell membranes. Significant LDH release is seen at 20 ug/mL and 50 ug/ mL, as shown by increased fold change values, indicating membrane damage and cytotoxicity. The positive control shows the highest LDH release, confirming maximum toxicity. The nanoparticles cause cell membrane damage at concentrations above 10 g/mL.

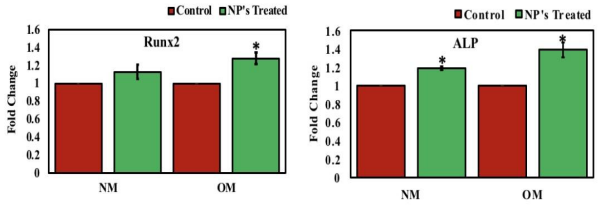

Figure 4: Changes in Runx2 ALP and Co—I mRNA expression in response to NP's treatment under osteogenic medium for 7d was assessed by qPCR analysis. *- indicates significant increased compared to the untreated control. The experiment was performed in biological replicates (n=3).

Runx2: No significant difference between control and NP-treated cells in NM. In OM, NP-treated cells show increased Runx2 expression, indicating enhanced osteogenic differentiation.

ALP: Similar trends as Runx2. NP-treated cells exhibit significantly higher ALP expression in OM, supporting osteogenic potential.

The nanoparticles promote osteogenic differentiation, particularly in an osteogenic medium.

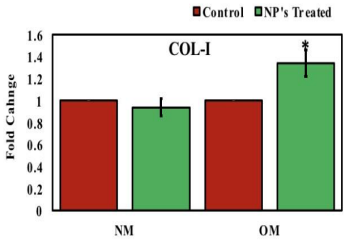

Figure 5: Changes Col-I mRNA expression in response to NP's treatment under osteogenic medium for 7d was assessed by qPCR analysis. *- indicates significant increased compared to the respective untreated control. The experiment was performed in biological replicates (n=3).

No significant difference in COL-I expression between control and NP-treated cells in NM. In OM, NP-treated cells show significantly increased COL-I expression, suggesting enhanced osteogenic activity. The nanoparticles upregulate COL-I expression in osteogenic conditions, further supporting their role in promoting osteogenesis.

Discussion:

In the field of bone tissue engineering, several types of nanoparticles have been tested due to their potential in encouraging osteogenic differentiation of MSCs. Our research on $CuZnFe_2O_4$ nanoparticles is consistent with evidence from other similar studies, where it provides information on their relative efficacy as well as mechanisms.

Iron oxide nanoparticles (Fe_3O_4) have been intensively studied for their great biocompatibility and for their osteoinductive properties. For example, Fe_3O_4 magnetic nanocomposites are found to protect MSCs and induce osteogenic differentiation while increasing the expression of osteogenic markers like Runx2 and ALP (H. Zhang et al. 2020). Similarly, hydroxyapatite-coated Fe_3O_4 nanoparticles markedly induced the production of ALP, collagen, and calcium in osteoblasts. Such results indicate the role of these nanoparticles in the bone regeneration process (Yang et al. 2022). Our findings on $CuZnFe_2O_4$ nanoparticles show similar upregulation of these markers and point to a similar mechanism by which they induce osteogenesis.

ZnO nanoparticles have also been shown to stimulate osteogenic differentiation. Research shows that concentrations up to 25 μg/mL of ZnO nanoparticles increase the osteogenic differentiation of rat bone marrow-derived MSCs by increasing calcium deposition, ALP activity, and osteogenic marker gene expression (S. Wang et al. 2024). In our study, $CuZnFe_2O_4$ nanoparticles, with zinc incorporated, also stimulated osteogenic differentiation, which could take advantage of the known osteoinductive effects of zinc ions.

The incorporation of multiple metal ions in nanoparticles can lead to synergistic effects on osteogenesis. For instance, hydroxyapatite whiskers modified with nano-ZnO have been demonstrated to enhance osteogenic activity by increasing ALP activity and calcium deposition (Wei et al. 2024). Our $CuZnFe_2O_4$ nanoparticles, which contain copper, zinc, and iron, may have a combined effect, which could enhance osteogenic differentiation through several pathways.

Activation of the signaling pathways Wnt/β-catenin and BMP/Smad is also critical in osteogenic differentiation. Nanoparticles, such as Fe_3O_4, have been reported to modulate these pathways and enhance osteogenesis.(B. Zhang et al. 2025) Although our study did not measure the activation of these pathways directly, the upregulation of markers for osteogenic differentiation by $CuZnFe_2O_4$ nanoparticles indicates the influence of these nanoparticles on the related signaling pathways, and the effects should be further explored.

Cytotoxicity is a significant concern in the use of nanoparticles for biomedical applications. The research on cobalt ferrite nanoparticles has shown that they are less cytotoxic at lower concentrations, and only at higher doses do they have significant effects (Nadia et al. 2024). Our results are in agreement with this as $CuZnFe_2O_4$ nanoparticles were biocompatible up to 10 μg/mL, but cytotoxic effects were apparent at higher concentrations .

Surface modification of nanoparticles can further improve their biocompatibility and osteoinductive properties. For example, Fe_3O_4/BSA particles have been demonstrated to induce osteogenic differentiation of MSCs under static magnetic fields (Jiang et al. 2016). It is likely that similar surface functionalization approaches for $CuZnFe_2O_4$ nanoparticles may further improve their efficacy in bone tissue engineering applications (Večerić-Haler et al. 2022).

Overall, our $CuZnFe_2O_4$ nanoparticles work well, and their effectiveness in enhancing the osteogenic differentiation of MSCs was consistent with other metal oxide nanoparticles research. Their application may also relate to the involvement of more than one metal ion in their synthesis process, potentially through activation of key signaling pathways.

Limitations:

1.Only *In-Vitro* studies: The existing work is restricted to *In-Vitro* MSC studies. Although the *In-Vitro* experiments are important for cytotoxicity, differentiation of osteogenic type of MSCs, the *in -vivo* behavior of nanoparticles in an extensive, complex physiological environment for such factors as immune reaction, systemic toxicity, and overall dynamic bone healing has been poorly understood.

2.Characterization of Nanoparticles: The study lacks detailed physicochemical characterization of $CuZnFe_2O_4$ nanoparticles, such as their size, shape, surface charge, and stability in biological media, which largely influence cellular uptake, toxicity, and osteogenic potential.

3.Limited Gene Expression Panel: Only three osteogenic markers, namely Runx2, ALP, and Col-I, were considered. Although these are very critical markers of osteogenesis, consideration of other markers, for example, OCN (osteocalcin), BMP2 (bone morphogenetic protein-2), or SPP1 (osteopontin), would be important in providing a better view of osteogenic differentiation.

4. Single-Dose Range for Differentiation: The study considers a single dose (10 μg/mL) to evaluate osteogenic differentiation. Consideration of a higher concentration range may also elucidate the dose effects of the drug on osteogenesis.

5.Long-Term Effects: The study assessed osteogenesis within a 7-day timeframe. Nonetheless, bone formation is a long-term process, and longer culture durations may be able to provide more comprehensive information on late-stage differentiation and mineralization.

Future Scope:

1.*In-Vivo* Studies: Future studies should involve the validation of the osteogenic potential and biocompatibility of $CuZnFe_2O_4$ nanoparticles in animal models. This would allow for the assessment of the integration of nanoparticles with native bone tissue, immune response, and long-term safety.

2.Nanoparticle Optimization: Further studies should be conducted to surface functionalize or coat nanoparticles to enhance biocompatibility, stability, and targeted delivery to bone tissue. Functionalized nanoparticles may also facilitate osteogenic differentiation by encapsulating bioactive molecules like growth factors or peptides.

3. Multifunctional Applications: The study demonstrates the osteogenic potential of $CuZnFe_2O_4$ nanoparticles. Future work can be done to explore multifunctional applications such as combining osteogenesis with antibacterial or magnetic properties for advanced bone tissue engineering.

4. Mechanistic Insights: Investigating the cellular and molecular mechanisms of nanoparticle-induced osteogenesis would provide greater insights. For example, the signaling pathways Wnt/β-catenin, BMP/Smad, or MAPK might be analyzed to understand more specifically how $CuZnFe_2O_4$ nanoparticles affect osteogenesis.

5. Toxicity Profiling: Comprehensive genotoxicity, immunotoxicity, and long-term systemic toxicity studies are important for translating the nanoparticles into clinical applications.

6. 3D Culture Models: Expanding the study into 3D culture systems or organoids can better mimic the native bone environment and may provide more physiologically relevant insights into nanoparticle interactions.

7. Controlled Release Systems: Further expanding their therapeutic efficacy for bone regeneration, $CuZnFe_2O_4$ nanoparticles can be explored as carriers for controlled release of osteogenic drugs or growth factors.

8. Clinical Translation: In bridging the gap between preclinical studies and clinical applications, regulatory requirements must be addressed and nanoparticle formulations optimized for scalable production and reproducibility for their use in bone regeneration therapies.

Expanding the scope and overcoming the limitations of the study will open the way to innovative practical solutions in bone tissue engineering and regenerative medicine.

Conclusion:

This article presents the first ever, although a robust, evaluation on both the biocompatibility profile and osteogenic potential of $CuZnFe_2O_4$ nanoparticles for utility in bone regeneration. The MTT assay results also indicated that these nanoparticles had no negative impact on cells up to 10 μg/mL at both early (24 h) and late (72 h) time compounds with cell viability comparable to untreated controls. Higher concentrations (≥20 μg/mL) caused substantial cytotoxicity as evidenced by diminished mitochondrial activity and enhanced LDH secretion, which serves as a marker for damage to the plasma membrane. Such findings emphasize the need for careful optimization of the nanoparticle concentration in biomedical applications to ensure adequate safety.

Besides the fact that they are biocompatible , $CuZnFe_2O_4$ nanoparticles were able to accelerate osteogenic differentiation significantly. The expression levels of main osteogenic markers (Runx2,ALP,COL-I) were significantly up-regulated when treated with the nanoparticles under osteogenic conditions. Runx2 plays a central role in early osteogenic differentiation and ALP and COL-I have been implicated as key proteins for matrix mineralization and bone tissue formation. These markers have been upregulated in presence of nanoparticles which not only sustain the cellular viability but directly stimulate the mesenchymal stem cells in osteogenic environments with osteogenic stimulation.

It could be concluded that, $CuZnFe_2O_4$ nanoparticles are highly promising for applications as biomaterials for bone tissue engineering. Therefore, their potential to sustain cell viability at scientifically relevant concentrations and their osteoinductive properties suggest them as a compelling candidate with high potential for applications in bone regeneration and repair. Nevertheless, the safety and efficacy must be confirmed through additional in vivo experiments as well as long-term biocompatibility tests prior to engagement in a clinical arena.

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