Deciphering the Role of Copper-Zinc Alloy Nanoparticles on Osteoblast Viability and Proliferation

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**Abstract:** Copper zinc alloy nanoparticles have gained significant attention in biomedical research due to their unique properties and potential applications in various fields, including tissue engineering and regenerative medicine. Understanding their effects on osteoblast viability and proliferation is crucial for assessing their suitability for bone regeneration therapies. This study aimed to investigate the role of copper-zinc alloy nanoparticles in promoting osteoblast viability and proliferation. Osteoblasts were cultured in vitro and exposed to varying concentrations of copper-zinc alloy nanoparticles. Cell viability was assessed using MTT assay, while cell proliferation was determined by counting the number of cells over time. Additionally, morphological changes and cell adhesion were evaluated using microscopy techniques. Cu-Zn nanoparticles were tested for their biocompatibility using indirect MTT assay on osteoblasts for 24 hours and they were found to be cyto-friendly with no alteration in the metabolic activity. Toxicity was observed beyond 50% conditioned medium exposure to cells and exhibited a drastic change in the metabolic activity indicating that the particles were toxic beyond this concentration.The particles exhibited a similar trend of toxicity by altering the cell membrane integrity leading to the release of LDH from the osteoblasts which is evident via LDH measurements. Therefore it was concluded that Cu-Zn at 50% was found to be non-toxic osteoblasts. These particles at this concentration also promoted the cell proliferation following 2d of exposure indicating its mitogenic nature. In conclusion, the research on deciphering the role of copper zinc alloy nanoparticles on osteoblasts viability and proliferation has provided valuable insights into their potential effects on bone health and regeneration. The findings of this study contribute to our understanding of the interactions between these nanoparticles and osteoblasts, and their implications for bone tissue engineering and regenerative medicine.

**Keywords:** Regenerative medicine, copper-zinc alloy, osteoblast proliferation, MTT assay, LDH assay

# INTRODUCTION

Osteoblast plays a vital role in bone formation and maintenance, and any disruption in their viability or proliferation can lead to impaired bone healing and skeletal disorders. [(Dirckx et al., 2013)](https://paperpile.com/c/S3CTNo/LtSA) Osteoblasts contribute to the maintenance of bone density and strength proper viability and proliferation ensure continuous renewal and reinforcement of bone, preventing conditions like osteoporosis.[(Aparna et al., 2021; Poornima et al., 2021; P. Verma & Muthuswamy Pandian, 2021)](https://paperpile.com/c/S3CTNo/PzsL8+hWQQf+RM2Aj) To evaluate their potential as a biomaterial for the engineering of bone tissue, it is therefore of the utmost importance to comprehend how osteoblast viability and proliferation are affected by copper-zinc alloy nanoparticles[(Sugumaran et al., 2023; “The Roles of Immune Cells in Bone Healing; What We Know, Do Not Know and Future Perspectives,” 2016)](https://paperpile.com/c/S3CTNo/s2M6+4KJk).

The creation of biomaterials that can support cell survival, proliferation, and differentiation in order to promote effective bone regeneration is essential for the success of bone tissue engineering[(Aparna et al., 2021; Poornima et al., 2021; P. Verma & Muthuswamy Pandian, 2021)](https://paperpile.com/c/S3CTNo/PzsL8+hWQQf+RM2Aj)

Due to their distinct physicochemical characteristics and capacity to interact with cells at the nanoscale, metallic nanoparticles have drawn a lot of interest as prospective candidates for these kinds of applications. [(N. Verma & Kumar, 2019)](https://paperpile.com/c/S3CTNo/KRAl). Researchers can determine whether these nanoparticles are suitable for promoting bone regeneration by deciphering the role they play in the behavior of osteoblasts. The effects of metallic nanoparticles on cell viability, proliferation, and differentiation have been the subject of numerous studies [(“Bio-Composite Scaffolds Containing Chitosan/nano-Hydroxyapatite/nano-Copper–zinc for Bone Tissue Engineering,” 2012)](https://paperpile.com/c/S3CTNo/tzBv) [(“Nanotechnology in Bone Tissue Engineering,” 2015)](https://paperpile.com/c/S3CTNo/5KAg).

Bone tissue engineering and regenerative medicine will benefit greatly from knowing how osteoblast viability and proliferation are affected by nanoparticles made of copper zinc alloy.[(Aparna et al., 2021; Poornima et al., 2021; P. Verma & Muthuswamy Pandian, 2021)](https://paperpile.com/c/S3CTNo/PzsL8+hWQQf+RM2Aj) New biomaterials that enhance osteoblast function and promote bone regeneration could benefit from this study's findings. In addition, future research efforts can be guided by a comprehensive comprehension of the cellular responses to copper zinc alloy nanoparticles in designing more efficient strategies for bone tissue engineering and enhancing the properties of nanoparticles.[(“Nanoscale Hydroxyapatite Particles for Bone Tissue Engineering,” 2011; Sugumaran et al., 2023)](https://paperpile.com/c/S3CTNo/r9kH+4KJk)

Copper and zinc, both essential trace elements have been shown to promote osteogenic differentiation, the process by which osteoblast differentiate to form a new bone. When combined in alloy nanoparticle form, copper and zinc synergistically enhance this effect leading to increased osteogenic potential[(Seo et al., 2010)](https://paperpile.com/c/S3CTNo/g9K1). Copper zinc alloy nanoparticles are also known to have angiogenic properties which is crucial for bone regeneration as it facilitates the delivery of oxygen, nutrients and other essential factors.

Copper's antimicrobial and angiogenic properties have been shown to speed up wound healing and promote bone regeneration.[(Jain & Verma, 2022; Marya et al., 2022)](https://paperpile.com/c/S3CTNo/SLB4t+D4q2r) On the other hand, cellular processes like gene expression, differentiation, and proliferation all depend on zinc. Utilizing the advantageous properties of both copper and zinc in an alloy nanoparticle form to enhance bone regeneration presents an opportunity.[(“Copper-Based Biomaterials for Bone and Cartilage Tissue Engineering,” 2021)](https://paperpile.com/c/S3CTNo/Hw9h)

However, little is known about how osteoblasts are affected by nanoparticles made of copper zinc alloy.[(Merchant et al., 2022; Pandiyan et al., 2022)](https://paperpile.com/c/S3CTNo/UAtfz+Uu1MS) To evaluate their potential to promote bone regeneration, it is essential to clarify their influence on osteoblast proliferation and viability.[(Chokkattu et al., 2022; Ramamurthy et al., 2022)](https://paperpile.com/c/S3CTNo/Ya0v7+weeAp)

By examining the effects of copper-zinc alloy nanoparticles on osteoblast proliferation and viability, this study aims to fill this knowledge gap. Additionally, the interactions that the nanoparticles have with osteoblasts can be better understood by looking at the morphological changes that they cause.

# MATERIALS AND METHODS

Copper zinc alloy nanoparticles were purchased from Sigma Aldrich limited. MG- 63 cells used for the study were procured from NCCS Pune, India. LDH kit, MTT reagent and Other reagents used in the study were purchased from Merck Millipore.

## Cell line maintenance

Human MG-63 cells were cultured in 10% FBS containing DMEM and 5% CO2 under standard culture conditions. After that, the cells were trypsinized and passed to the appropriate numbers. After that, additional experiments were carried out with the subcultured cells.

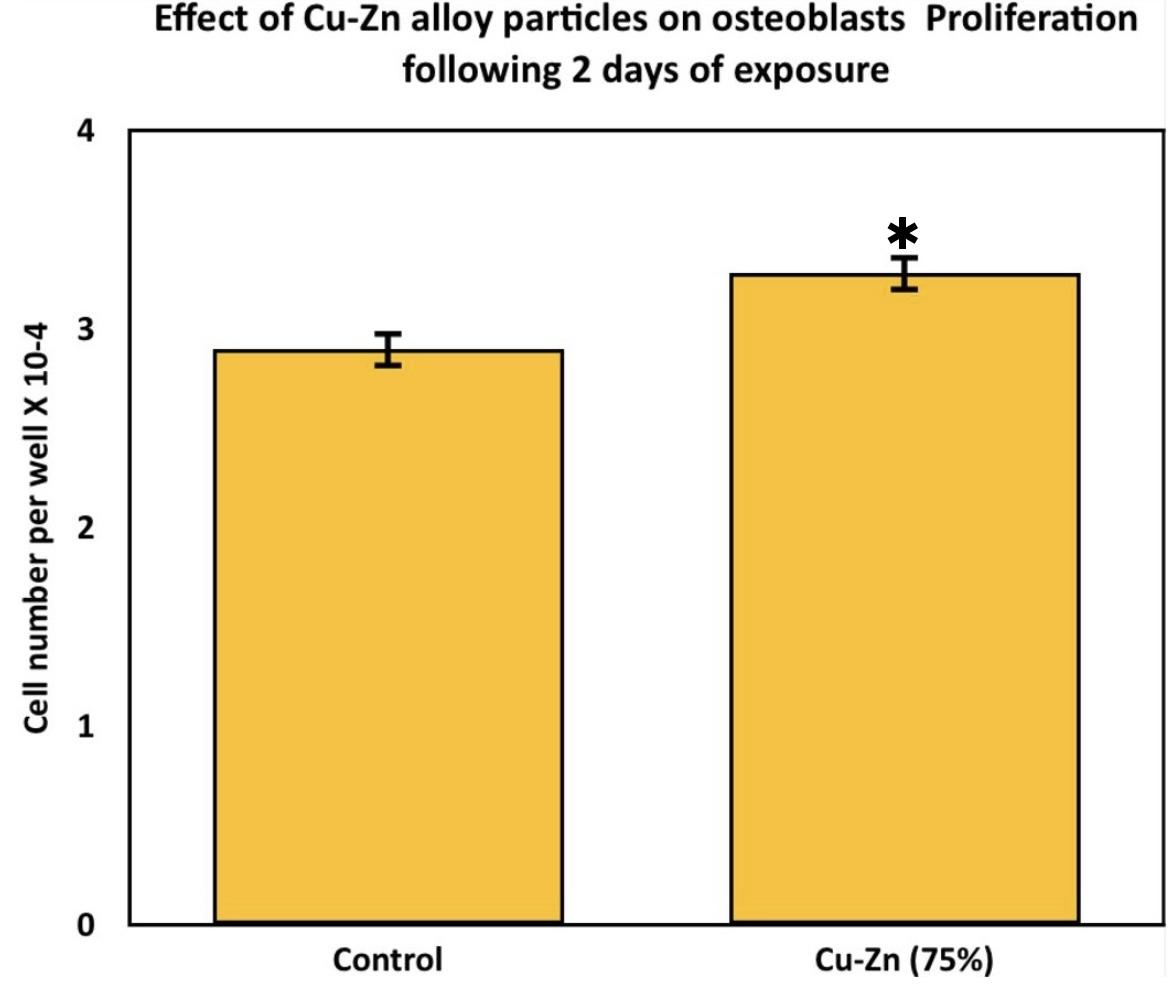
## MTT assay and LDH assay

After being trypsinized, MG-63 cells were aliquoted into the 24 well plates at 2 105 cells ml-1. For 24 hours, cells were exposed to various concentrations of Cu-Zn alloy nanoparticles conditioned medium (25% to 100%) in appropriate volumes of expansion medium. For the MTT assay, cells treated with 0.1% Triton-X 100 served as a positive control, while for the LDH assay, cells treated with 100 M H2O2 served as a positive control. After 48 hours of incubation, the conditioned medium taken from each well was measured for LDH release. The assay kit protocol was used to calculate the percentage of LDH release.

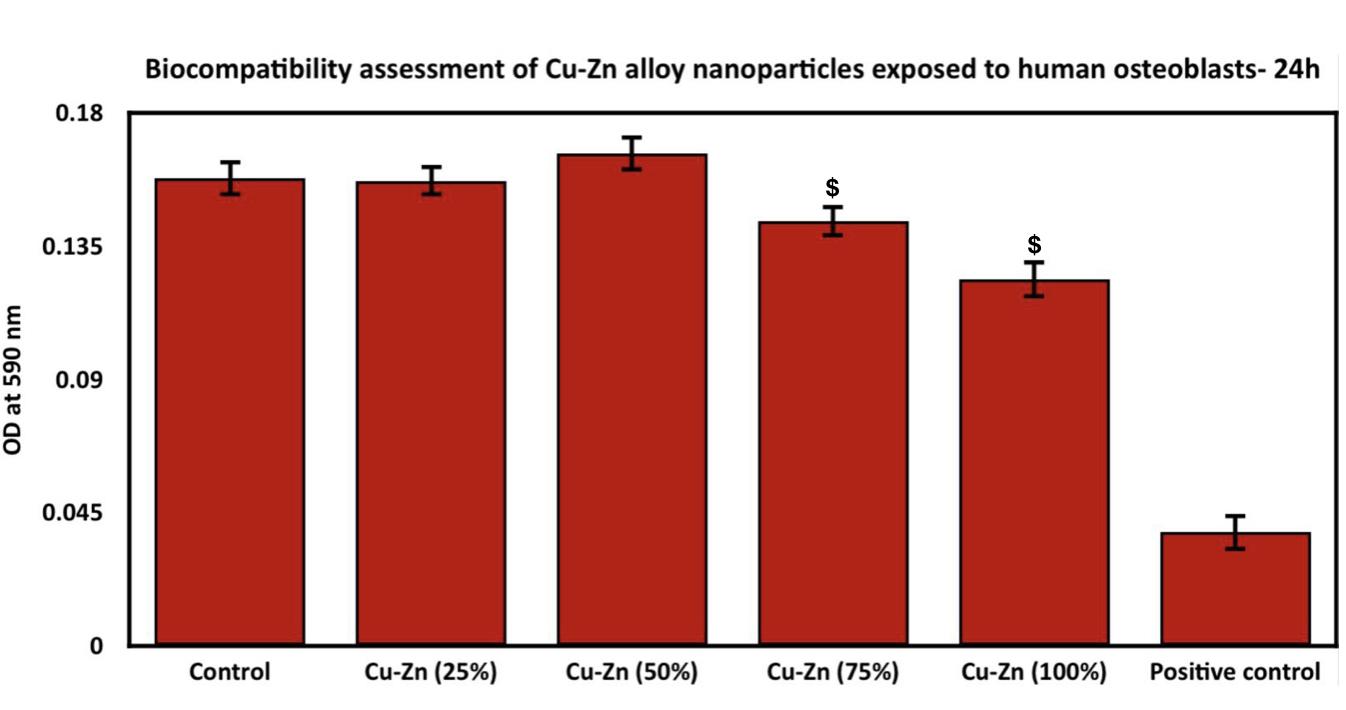
## Cell count using trypan blue exclusion assay

All of the samples were given to the cells for 24 hours at the nanoparticle concentrations of 20%, 50 %, 75%, and 100% respectively. Live-dead cell counts were performed using the Trypan blue exclusion assay. 500 uL of cells were incubated for three minutes at room temperature with an equal volume of 0.4% (w/v) trypan blue solution made from 0.81 percent NaCl and 0.06 percent (w/v) dibasic potassium phosphate for trypan blue staining. Using a light microscope and a four-chamber culture slide (BD Falcon CultureSlides), the number of cells in each chamber was counted. To determine the proportion of live and dead cells, 100 non-viable and viable cells were separately recorded.

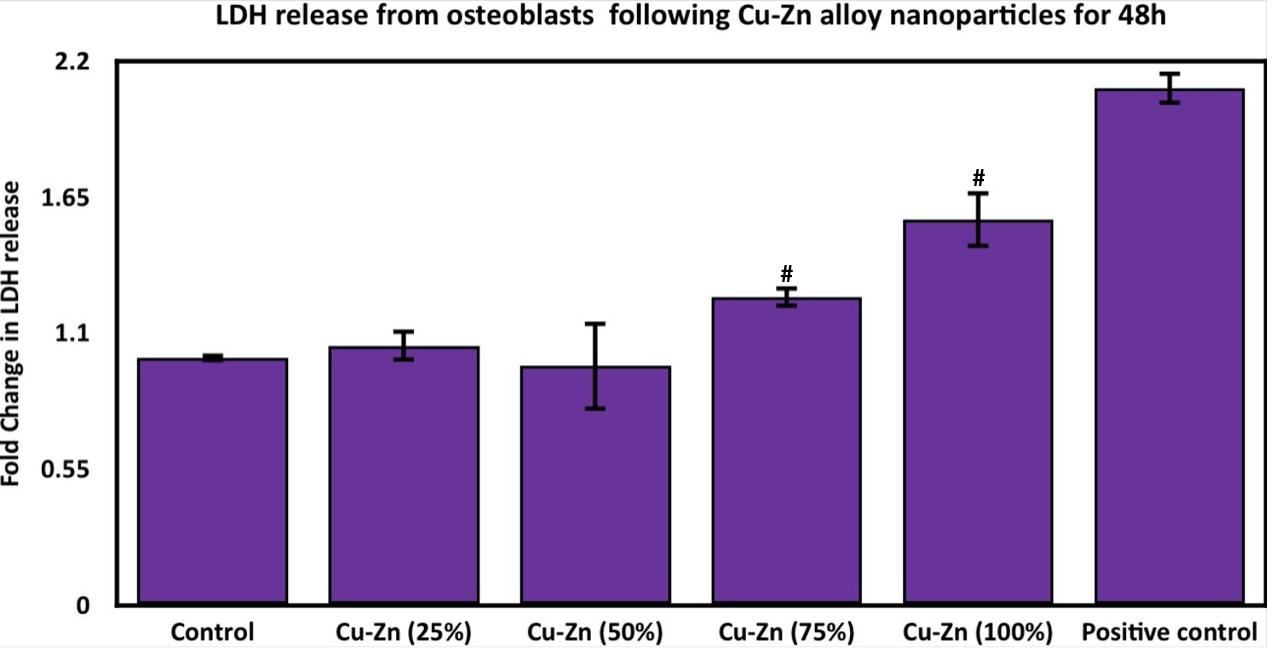
# RESULTS



**FIGURE 1:** Represents the effect of Cu- Zn alloy particles on osteoblast proliferation following 2 days of exposure . The x - axis represents the control group and the concentrations of Cu-Zn alloy nanoparticles and the y- axis represents the cell number per well x 10-4 . It was noted that at 75% concentration the effect of Cu-Zn alloy particles on osteoblast proliferation showed an increase when compared to the control group



**FIGURE 2:** Representing biocompatibility assessment of Cu-Zn alloy nanoparticles exposed to human osteoblast for 24 hours . The x - axis represents the control group and the various increase in concentrations of Cu-Zn alloy nanoparticles and the y- axis represents the OD at 590 nm . It was noted that the cell viability was highest in 50% concentration and a decrease was noted in increasing concentration (represented by $) indicating that the Cu-Zn alloy nanoparticles are biocompatible and safe at 50 % concentration



**FIGURE 3:** Represents the LDH release from osteoblast following Cu-Zn alloy nanoparticles for 48 hours . The x - axis represents the control group and the various increase in concentrations of Cu-Zn alloy nanoparticles and the y- axis represents the fold change in LDH release . It was noted that injury to the cell (#) was seen in Cu-Zn 75% and Cu-Zn 100 % concentrations respectively and the safest concentration was noted to be at 50%

Copper zinc alloy nanoparticles are widely used for biomedical applications, especially in bone tissue regeneration. However, the ability of these particles in increasing osteoblasts proliferation is still at its infancy. Enhancing bone formation during normal bone remodeling can be achieved by promoting the proliferation of osteoblasts or stimulating their differentiation. Osteoblasts play a crucial role in the formation of new bone, while osteoclasts are primarily responsible for bone resorption. In the field of bone tissue engineering, biomaterials need to possess several key properties in order to be effective. They should exhibit high biocompatibility, possess mechanical rigidity, and be biodegradable. Additionally, the surface topography and charge of the biomaterials are important factors that influence their ability to promote bone regeneration. These fundamental characteristics play a significant role in facilitating cellular activities such as adhesion, migration, proliferation, and the differentiation of cells into osteogenic lineages.

Initially,we have assessed the proliferation of osteoblast following two days of exposure and cell proliferation was observed in 75% concentration of Cu-Zn (Figure 1) and then we assessed the non-toxic dose of these particles at two levels (a) Metabolic activity and (b) cell membrane integrity (Chehelgerdi et al., 2023). Human osteoblastic cells were treated with different amounts of conditioned medium obtained from Cu-Zn alloy nanoparticles for a period of 24 and 48 h. Following treatment, the MTT results indicated that cells experienced significant toxicity after 50% of the conditioned medium. There was no alteration in the metabolic activity of cells till 50% of the conditioned medium (Figure 2). Similarly, there was no significant increase in the LDH release till 50% while it drastically increased in a dose-dependent manner ( Figure 3 )

# DISCUSSION

Metallic nanoparticles (NPs) have a higher dissolution rate compared to their bulk counterparts, resulting in the release of metallic ions that can be toxic when present in excessive amounts within the body.[(Adel et al., 2023)](https://paperpile.com/c/S3CTNo/OUQlF)Specifically, in the case of copper, these ions can displace trace metals bound to proteins, thereby rendering them inactive or causing their degradation. Additionally, the redox cycle between Cu1+ and Cu2+ generates an elevated level of reactive oxygen species (ROS), both inside and outside the cells.[(Subramanian & Harikrishnan, 2023)](https://paperpile.com/c/S3CTNo/5XNtC)

To further understand the processes underlying this increased cell viability and proliferation, more research is required.[(Laghari et al., 2023; Ramakrishnan et al., 2023)](https://paperpile.com/c/S3CTNo/aRUHb+K7sA8).Long-term evaluations and in vivo research will also be required to fully comprehend the efficacy and safety of these conditions for use in real-world applications (Saadh et al., 2024). Similar studies have been conducted in relation to our study using other nanoparticles on cell lines and have been discussed below .

Recent developments in materials science and nanotechnology have sparked a great deal of interest in metallic and metallic oxide nanoparticles because they provide a wide range of solutions to the challenges facing the orthopedic sector.[(Muthuswamy Pandian et al., 2022)](https://paperpile.com/c/S3CTNo/78xbO) More significantly, these nanoparticles can be added to orthopedic implants and scaffolds to improve their antimicrobial capacity, bioactive molecular delivery, mechanical strength, osseointegration, and cell labeling and imaging. These properties are exclusive to these nanoparticles and are not present in conventional materials. But a lot of metallic and metallic oxide nanoparticles can potentially be harmful to the tissues and cells in close proximity. [(Elakkiya et al., 2023; Wang et al., 2021)](https://paperpile.com/c/S3CTNo/SDQf+RKWo)

In a study by Yanhua Hou et al it was suggested that In a dose- and size-dependent way, the TiO2 nanoparticles negatively impacted MSC cell survival, proliferation, and the cell cycle.[(Muthuswamy Pandian et al., 2022; Ramakrishnan et al., 2023)](https://paperpile.com/c/S3CTNo/78xbO+aRUHb) The effects of particle internalization on MSC adherence, spreading, and morphology were examined using confocal laser scanning microscopy. Large TiO2 nanoparticles adversely affected the integrity of MSC's cell membrane, cytoskeleton, and vinculin.[(Hou et al., 2013)](https://paperpile.com/c/S3CTNo/G8DX)

In another study Howa Begum et al suggested that Osteoblast (MG63) cell culture on both pure and zinc-doped HAp blocks, made using the slip casting method and sintered at various temperatures, showed that the doped specimens responded better.[(Ganapathy 2021)](https://paperpile.com/c/S3CTNo/iOl4e) According to an XRD analysis, the zinc dopant altered the hydroxyapatite crystal's lattice parameter in proportion to the respective ion sizes of calcium and zinc. Doped samples have improved rates of cell survival, adhesion, and proliferation (especially with greater sintering temperature)[(“MG63 Osteoblast Cell Response on Zn Doped Hydroxyapatite (HAp) with Various Surface Features,” 2017)](https://paperpile.com/c/S3CTNo/onqH)

In a study conducted by Ateka Khader et al in 2019 MSC differentiation was assessed based on biocompatibility. The lowest Zn-NP concentrations encouraged chondrogenic development, as evidenced by the highest expression of genes related to cartilage and the formation of collagen type II. Conversely, human MSC osteogenic differentiation was enhanced by large quantities of Zn-NPs. The highest levels of ALP activity, collagen synthesis, and expression of genes unique to bones demonstrated this differentiation. The study's findings demonstrate Zn-NPs composite scaffolds' potential for bone tissue engineering. [(Khader & Arinzeh, 2020)](https://paperpile.com/c/S3CTNo/Hg5a)

As a result of their biocompatibility, capacity to stimulate migration of endothelial cells and proliferation, and antioxidant properties, copper nanoparticles (Cu-NPs) are useful for wound healing[(Ghosh & Webster, 2021; Tangri et al., 2023)](https://paperpile.com/c/S3CTNo/58l8+fqwv). Bone tissue regeneration can be enhanced by the incorporation of copper ions in scaffolds. D'Mello et al. examined how copper-doped carbon scaffolds affected bone regrowth. In male Fisher 344 rats, scaffolds were implanted into 5 mm calvarial defects. After four weeks, histological analysis and micro-CT scans revealed an increase in bone volume. They came to the conclusion that adding copper ions to scaffolds might improve tissue regeneration.[(D’Mello et al., 2015)](https://paperpile.com/c/S3CTNo/w9PC)

After 2 day exposure of the osteoblast cells to the Cu-Zn alloy nanoparticles MTT assay was conducted to assess the biocompatibility and LDH release from osteoblast was noted . The results were compared with the studies done previously by other authors and it can be concluded that the Cu - Zn alloy nanoparticles can help in proliferation of osteoblasts and was also found to be biocompatible at a certain concentration . Due to limited availability of time and resources only in vitro studies were performed and performing inVivo studies will help understand the biocompatibility and safety of the material more precisely

# CONCLUSION

The results of our study suggest that copper-zinc alloy nanoparticles may have the ability to speed up the healing process in orthopedic and dental applications by enhancing bone regeneration. Addition of copper zinc alloy nanoparticles proliferated the growth of the osteoblasts . To assess the biocompatibility MTT assay was conducted and it was found that copper zinc alloy nanoparticles are highly biocompatible at 50% of conditioned medium .

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