

## BIOCOMPATIBILITY AND OSTEOGENIC PROPERTY OF COPPER NANOPARTICLES

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**Abstract:** Copper ions are known to promote the process of angiogenesis. Numerous previous investigations have shown that the angiogenic potential of biomaterials is increased when copper salts are added. Numerous orthopaedic and dental implants that help patients lead better lives have been used copper nano particles. Copper nanoparticles were dispersed in sterile distilled water and ultrasonicated for 30 minutes to obtain a homogenous suspension. Different concentrations (5, 10, 25, 50, and 100 µg/mL) were prepared for cellular studies. MTT assay, LDH leakage assay, DCF-DA ROS assay, RUNX2, ALPGOLI-1 and OCN were analysed. MTT assay, LDH leakage assay, DCF-DAROS assay, RUNX2, ALPGOLI-1 and OCN were analysed. these analysis showed increased activity. It is concluded that copper nanoparticles possess potent osteogenic and biocompatibility. It can be made into a better graft or an implant which requires osteogenicity and biocompatibility in further research

**Keywords:** nanoparticles, health, osteogenicity, quality adjusted life year, biocompatibility, illness.

### 1. Introduction

The scientific study of and use of nanotechnology has advanced significantly in recent years(1). The fascinating and astonishing characteristics of nanoparticles, such as their durability, high diffusivity, flexible chemical and biological activities, and also their variety of applications when compared to their bulk components, have led to an exponential growth in the field of nanotechnology(2,3). The enormous potential of nanotechnology has many uses in medicine. It has led to the use of biology and nanoscience in the fight against infectious diseases and against disorders(4). Particularly, the production of nanoparticles using eco-friendly, green nanotechnology-based technologies has drawn a lot of attention globally(5).

Numerous orthopaedic and dental implants that help patients lead better lives have been solutions to this problem to rapid developments in material science and medicine(6). Through the use of synthetic grafts, bone abnormalities caused by trauma, illness, or congenital disorders can be completely repaired and replaced. This is the goal of bone tissue engineering(7,8). Osteoprogenitor cell division, structure, and proliferation are vital for tissue development. The development of blood vessels through angiogenesis, where the delivery of oxygen and nutrients facilitates bone rebuilding at the defect location, gives tissue grafts some life(9). Therefore, angiogenesis is essential for an implanted graft to be successful(10). Fracture non-union is a common clinical disease caused by the absence of blood vessel development in the bone fracture callus. The fracture site is where this illness is detected, and therapy involves promoting angiogenesis and repeated procedures(11). Copper ions are known to promote the process of angiogenesis(12). In order to prevent angiogenesis and slow the formation of tumors, copper chelators have been explored as anti-tumor medications in recent years(13,14). Copper ions released from copper doped bioglass promote angiogenesis (15). Numerous previous investigations have shown that the angiogenic potential of biomaterials is increased when copper salts are incorporated into mesoporous bioactive glass scaffolds or bioglass/polymer composite scaffolds (16). In a recent study, copper nanoparticles were discovered to have more angiogenic activity than copper salts(17). The infection of having placed an implant is a significant issue that frequently requires expensive treatment. Due to their high surface-to-volume ratio, copper nanoparticles have been shown to be extremely toxic to a wide range of bacteria and fungi(18). These particles typically kill cells by a variety of mechanisms, including membrane disruption, blocking biochemical pathways, complex formation with proteins, and DNA damage(19). Which make copper nanoparticles a better antibacterial agent and protect tissue surrounding the implant against infectious pathogens(20). Therefore the study aims to analyze the biocompatibility and osteogenic properties of copper nanoparticles.

### 2. MATERIALS AND METHOD

The following chemicals and reagents were used in the present study:

- Copper nanoparticles (CuNPs) (20–80 nm)
- Dulbecco's Modified Eagle Medium (DMEM)
- Fetal Bovine Serum (FBS)
- Penicillin–Streptomycin solution
- Trypsin–EDTA
- Phosphate Buffered Saline (PBS)
- Human osteoblast-like MG-63 cells/human mesenchymal stem cells (hMSCs)
- MTT reagent
- Lactate Dehydrogenase (LDH) Cytotoxicity Assay Kit
- 2',7'-Dichlorofluorescein diacetate (DCFH-DA)
- Osteogenic differentiation medium
- RNA isolation kit
- cDNA synthesis kit
- SYBR Green Master Mix
- Primers for RUNX2, ALP, COL-1, and OCN genes
- Dimethyl sulfoxide (DMSO)
- Paraformaldehyde
- Analytical-grade chemicals and distilled water

#### 2.1 Preparation of Copper Nanoparticles

Copper nanoparticles were dispersed in sterile distilled water and ultrasonicated for 30 minutes to obtain a homogenous suspension. Different concentrations (5, 10, 25, 50, and 100 µg/mL) were prepared for cellular studies.

**2.2 Cell Culture:** MG-63 osteoblast-like cells/hMSCs were cultured in DMEM supplemented with 10% FBS and 1% penicillin–streptomycin solution. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. The culture medium was changed every 2–3 days.

#### 2.3 MTT Assay for Cell Viability

The cytotoxicity and biocompatibility of copper nanoparticles were evaluated using the MTT assay.

1. Cells were seeded into 96-well plates at a density of  $1 \times 10^4$  cells/well and incubated for 24 hours.
2. Cells were treated with varying concentrations of copper nanoparticles for 24 and 48 hours.
3. After treatment, 20 µL of MTT solution (5 mg/mL) was added to each well and incubated for 4 hours.
4. The formed purple formazan crystals were dissolved using DMSO.
5. Absorbance was measured at 570 nm using a microplate reader.

Cell viability was calculated using:  $\text{Cell Viability (\%)} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of control cells}} \times 100$

#### 2.4 LDH Leakage Assay

Cell membrane integrity was assessed using the LDH leakage assay.

1. Cells were seeded in 96-well plates and treated with copper nanoparticles at different concentrations.
2. Following 24 hours of incubation, culture supernatants were collected.
3. LDH reagent was added according to the manufacturer's instructions.
4. The reaction mixture was incubated at room temperature for 30 minutes.
5. Absorbance was measured at 490 nm using a microplate reader.

The percentage of LDH leakage was calculated as:

$$\text{LDH Leakage (\%)} = \frac{\text{Experimental LDH Release}}{\text{Maximum LDH Release}} \times 100$$

**2.5 DCFH-DA ROS Assay**

Intracellular reactive oxygen species (ROS) generation was measured using the DCFH-DA assay.

1. Cells were seeded in 6-well plates and treated with copper nanoparticles for 24 hours.
2. Cells were washed with PBS and incubated with DCFH-DA dye (10 μM) for 30 minutes at 37°C in the dark.
3. Excess dye was removed by washing with PBS.
4. Fluorescence intensity was observed under a fluorescence microscope and quantified using a fluorescence microplate reader at excitation/emission wavelengths of 485/535 nm.

Increased fluorescence intensity indicated elevated intracellular ROS production.

**2.6 Osteogenic Gene Expression Analysis**

The osteogenic differentiation potential of copper nanoparticles was evaluated by analyzing the expression of RUNX2, ALP, COL-1, and OCN genes using quantitative real-time PCR (qRT-PCR).

RNA Isolation and cDNA Synthesis

1. Cells were cultured in osteogenic medium with copper nanoparticles for 7, 14, and 21 days.
2. Total RNA was extracted using an RNA isolation kit.
3. Purified RNA was reverse transcribed into complementary DNA (cDNA) using a cDNA synthesis kit.

**2.7 Quantitative Real-Time PCR (qRT-PCR)**

Gene expression analysis was performed using SYBR Green Master Mix with specific primers for:

- RUNX2
- ALP
- COL-1 (Collagen Type I)
- OCN (Osteocalcin)

GAPDH was used as the housekeeping gene.

**3. RESULT**

According to the findings of the study, it is found that MTT assay performed in C3H10T1/2 mouse mesenchymal stem cells have shown with increase in concentration there is increase in OD value ( 5, 10, 20, 50 ug/ml) at 570 nm (figure 1). The LDH assay performed in C3H10T1/2 mouse mesenchymal stem cells was increased to two fold at 50 ug/ml (figure 2). The DCF-DA ROS assay performed in C3H10T1/2 mouse mesenchymal stem cells for a period of 24 hrs, it indicated that there was an increase in relative fluorescence unit (figure 3). The expression of Runx2 in Real time PCR analysis of osteoblast differentiation marker genes. Mouse mesenchymal stem cells were grown under normal medium (NM) and osteogenic medium (OM) for 7 days, there was an increase in expression of RUNX2 gene 1.15 times(figure 4). The expression of Alkaline phosphatase enzyme in Real time PCR analysis of osteoblast differentiation marker genes. Mouse mesenchymal stem cells were grown under normal medium (NM) and osteogenic medium (OM) for 7 days, there was an increase in expression of ALP enzyme 1.2 times( figure 5). The expression of GOL-1 in Real time PCR analysis of osteoblast differentiation marker genes. Mouse mesenchymal stem cells were grown under normal medium (NM) and osteogenic medium (OM) for 7 days; it was to increase in expression GOL-1 gene 1.2 times(figure 6). The expression of OCN in Real time PCR analysis of osteoblast differentiation marker genes. Mouse mesenchymal stem cells were grown under normal medium (NM) and osteogenic medium (OM) for 7 days, there was an increase in expression of OCN 1.25 times (figure 7).

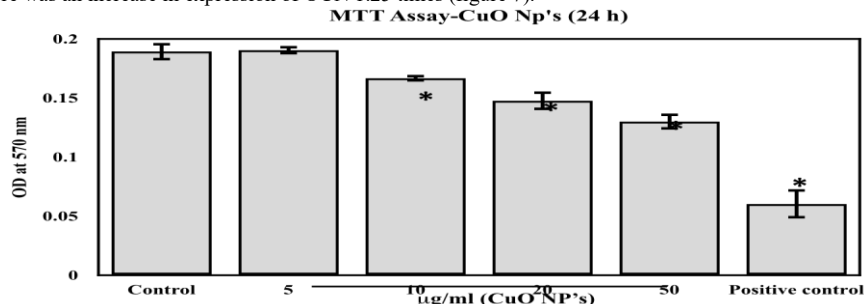


FIGURE 1: The graph depicts the MTT assay performed in C3H10T1/2 mouse mesenchymal stem cells.

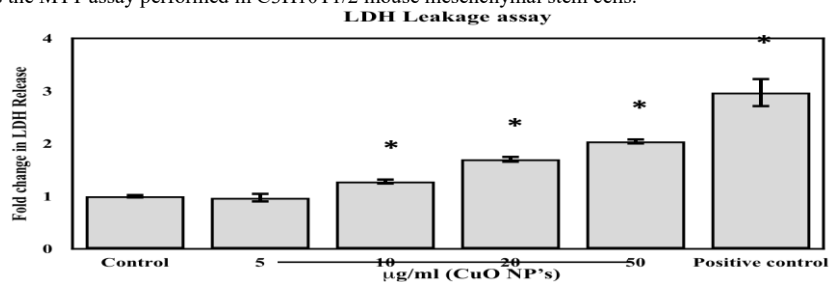


FIGURE 2: The graph depicts the LDH assay performed in C3H10T1/2 mouse mesenchymal stem cells.

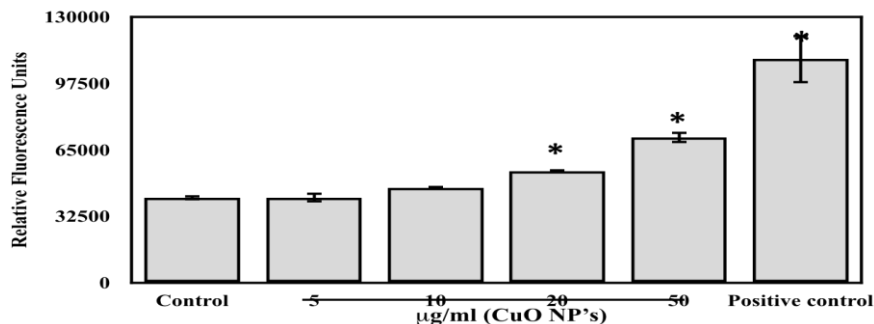
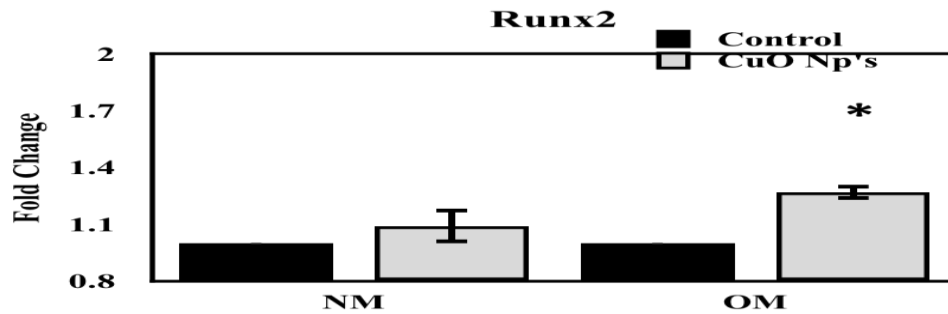
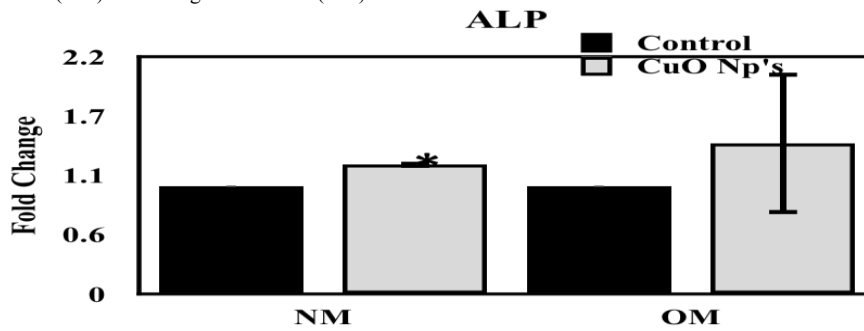


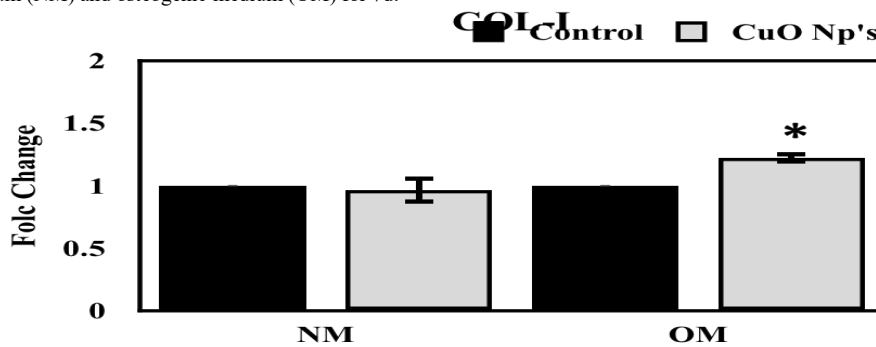
FIGURE 3: The graph depicts the DCF-DA ROS assay for performance in C3H10T1/2 mouse mesenchymal stem cells (24h).



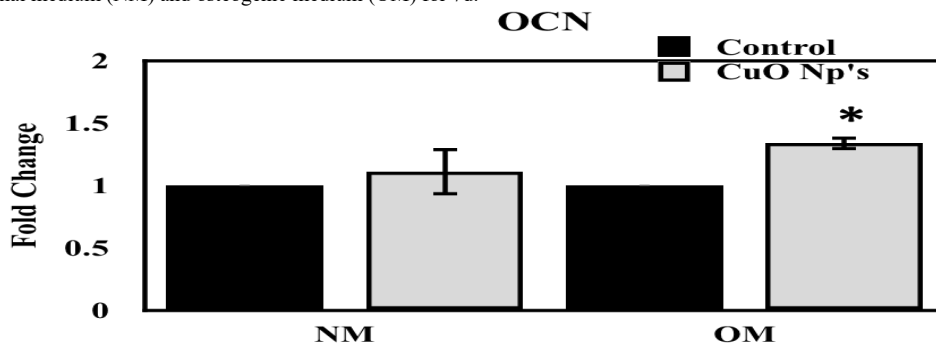
**FIGURE 4:** The graph depicts the expression of Runx2 in Real time PCR analysis of osteoblast differentiation marker genes. Mouse mesenchymal stem cells were grown under normal medium (NM) and osteogenic medium (OM) for 7d.



**FIGURE 5:** The graph depicts the expression of ALP in Real time PCR analysis of osteoblast differentiation marker genes. Mouse mesenchymal stem cells were grown under normal medium (NM) and osteogenic medium (OM) for 7d.



**FIGURE 6:** The graph depicts the expression of GOL-1 in Real time PCR analysis of osteoblast differentiation marker genes. Mouse mesenchymal stem cells were grown under normal medium (NM) and osteogenic medium (OM) for 7d.



**FIGURE 7:** The graph depicts the expression of OCN in Real time PCR analysis of osteoblast differentiation marker genes. Mouse mesenchymal stem cells were grown under normal medium (NM) and osteogenic medium (OM) for 7d.

#### 4. DISCUSSION

Angiogenesis, a complicated process that involves the growth of new blood vessels, is necessary for a scaffold's full regeneration. The developing vasculature supplies nutrition, growth factors, hormones, and other necessary proteins for the development of vascular tissue. When it comes to molecular and cellular signaling that encourages angiogenesis, VEGF is essential, effective, and a long-lasting signal(21). Osteogenicity and biocompatibility of a graft is important for the selection of implant materials(22). The copper nanoparticles synthesized have been found to satisfy the requirements for the manufacturing of dental implants. The copper nanoparticles had shown a better osteogenic and biocompatibility when compared to copper ions due to its high surface to volume ratio. The previous studies conducted by Cubillo et.al (18)utilized monodisperse copper nanoparticles in a matrix of sepiolite for antibacterial purposes. Sepiolite delayed the liberation of copper nanoparticles as well as prevented their aggregation. The functional groups, which were present in the culture filtrate, were in charge of the reduction reactions involving proteins or enzymes that resulted in the synthesis of CuO NPs. The FTIR spectra of CuO NPs contain exhibited vibrations in the areas between 400 and 4000  $\text{cm}^{-1}$ , which can be attributed to the vibrations of M-O (M=Cu), further verifying the CuO NPs production(23). The Joint Committee on Powder Diffraction Requirements (JCPDS) standards were met by the XRD examination of the CuO NPs made from actinomycetes, confirming their crystalline nature with a monoclinic phase (JCPDS No: 01-1117). All of the CuO NPs' peaks can be related to the C2/c space group's monoclinic CuO crystal system's crystallographic characteristics(24).In the osteogenic medium, the effect of copper eluting nanocomposites on mineralization was investigated on Day 14, when mineralization was quantified, it was discovered that PCL/RGO Cu 5 had an 87% higher level of mineralization than other nanocomposites. The differences between PCL/RGO and PCL/Cu are negligible. According to several studies, the Reduced graphene oxide Cu loaded nanocomposite scaf- folds' released copper ions appear to have encouraged osteogenesis(25). The ability of this composite comprising the hybrid particles to enhance angiogenesis is thus indicated by the increased expression

of the VEGF and ANG2 genes in cells on PCL/RGO Cu 5 composite. This may be due to the ongoing release of copper ions, which is anticipated to result in *in vivo* angiogenesis(26).

## 5. CONCLUSION

From the results obtained, it is concluded that copper nanoparticles possess potent osteogenic and biocompatibility. It can be made into a better graft or an implant which requires osteogenicity and biocompatibility in further research. It has been confirmed in experimental rats, showing increased RUNX2 enzyme, GOL-1 gene, ALP and OCN. It also showed better results in MTT assay and LDH leakage assay. Which in collective shows its osteogenic property. With further research it can be manufactured into a better implant.

## AUTHORS CONTRIBUTION:

Iammaman varshan contributed to literature survey, experimental work, data collection, analysis, and manuscript preparation.

Dr. Keerthi Sasanka L contributed to literature survey, experimental work, data collection, analysis, and manuscript preparation.

Dr. Saravana Sekaran supervised the study design, analysis, and manuscript correction.

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