Chapter 7  

In Vitro studies

7.1. Introduction

Copper, the magical element, has been reported since centuries for its significant role in treatment of various physiological disorders (Watts, 1989, Uauy et al., 1998, Prohaska, 2014).

Copper is used as antimicrobial agent since ages (Borkow and Gabbay, 2009). Copper nanoparticles (CuNps) show better antimicrobial activity than its native form (Hajipour et al., 2012). CuNps were found to be better antimicrobial agents (Ruparelia et al., 2008). Copper nanoparticles have shown antimicrobial effect by various mechanisms (Chatterjee et al., 2014). Biosynthesized copper nanoparticles (BCuNps) have been evaluated for their antimicrobial activity against different microorganisms (Abboud et al., 2014, Shende et al., 2015, Tiwari et al., 2014).

Copper is an essential element for our body and known to possess antimicrobial, anti-inflammatory and angiogenesis properties which makes it useful in wound healing (Sen et al., 2002, Borkow et al., 2010a). Copper peptides have shown improved wound healing in diabetic foot ulcer (Mulder et al., 1994, Konerding et al., 2012). Copper complex has also shown significant improvement in wound healing (Somayaji et al., 1995). CuNps were reported to improve wound healing (Rakhmetova et al., 2010). BCuNps and silver nanoparticles were evaluated earlier for their wound healing activity (Kalaiselvan and Rajasekaran, 2009, Tiwari et al., 2014).

Over exposure to copper has been reported for its cytotoxic, oxidative stress and genotoxic effect on human cells (Grillo et al., 2010). CuNps were found to be cytotoxic on various cells. CuNps have been reported to induce oxidative stress and cytotoxicity in lung epithelial cells (Fahmy and Cormier, 2009). CuNps were found to be genotoxic in lung epithelial cells (Ahamed et al., 2010).

Osteoporosis is a common disease in postmenopausal women (Ip et al., 2013). There is no effective osteoporosis treatment available till now even after supplementing with vitamin D and calcium (Mithal et al., 2014). It was reported that women with the osteoporosis, have copper deficiency (Strain, 1998, Chaudhri et al., 2009). Copper is one of the essential micronutrient which helps in bone metabolism. Being a cofactor for lysyl oxidase, it helps in cross-linking of collagen fibres in bone and also inhibits bone resorption by reducing the free radical with the help of superoxide dismutase (Uauy et al., 1998, Rayton and Harris, 1979). Copper deficiency could enhance bone abnormalities (Sierpinska et al., 2014). It has been
reported to stimulate cell growth and differentiation (Li et al., 2012, Milkovic et al., 2014). It has found to improve proliferation of osteoblast cells (Fiedler et al., 2011).

In the present work, we have evaluated in vitro effect of BCuNps for antimicrobial activity, cytotoxic effect, wound healing effect on skin keratinocytes cells (HaCat), anticancer effect on epithelial lung carcinoma cells (A549) and supplementation on human foetal osteoblast (hFOB). These biocompatible BCuNps were synthesized by a copper resistant isolate.

7.2. Methodology

7.2.1. Antimicrobial studies (Liu et al., 2007, Sarker et al., 2007, Jorgensen and Turnidge, 2015)

Antimicrobial studies of BCuNps were performed against Gram positive and Gram negative bacteria. These studies were performed to assess the antimicrobial activity of BCuNps in comparison with native copper (copper sulphate). Cell free filtrate of SWSD1 and ciprofloxacin were used as control and standard drug. Disc diffusion and micro dilution methods were used to evaluate the antimicrobial activity. McFarland standard (0.5) culture of following organisms, Escherichia coli (NCIM 5011), Bacillus subtilis (NCIM 2063), Pseudomonas aeruginosa (NCIM 2036) and Staphylococcus aureus (NCIM 2079) were prepared for these antimicrobial studies. BCuNps and native copper were used at similar concentration. Disc diffusion method was performed using spread plate technique. Micro dilution assay was performed with colorimetric estimation using resazurin dye.

7.2.2. Cell viability and cytotoxicity studies (Sankhe et al., 2015, Magcwebeba et al., 2012, Jain et al., 2013, Hapidin et al., 2015, Tiwari et al., 2014, Koganti et al., 2013)

Cell viability and cytotoxicity studies of BCuNps were performed against various mammalian cells lines. Studies were conducted with normal (HaCat, Vero, and hFOB 1.19) as well as cancerous (MCF7, A549, CaCO-2, Neuro 2a, HCT116, RAW 264.7 and HepG2) cell lines. Vero, MCF7, A549, CaCO-2, HCT 116 and RAW 264.7 were cultured with Dulbecco’s Modified Eagle’s Medium (DMEM). HaCat, hFOB 1.19, and Neuro-2a cell lines were cultured with 1:1 mixture of DMEM and Ham's F12 medium. HepG2 cell line was cultured with Minimum Essential Medium (MEM). These media were supplemented with 10 % Foetal Bovine Serum (FBS), penicillin (100 IU/mL), streptomycin (100 µg/ml) and amphotericin-B (5 µg/ml) for proliferation. For cell proliferation, all the cell lines, except hFOB cell line, were incubated at 37 °C in 5 % CO₂ humidified atmosphere. hFOB cell line was incubated at 33.5 °C in 5 % CO₂ humidified atmosphere. Cell viability and cytotoxicity studies were performed
in 96 well plate with different concentration of BCuNps in maintenance medium (proliferation medium with 2 % FBS). Native copper (copper sulphate) was used in similar concentration range to see the improvement in cell viability and cytotoxicity effects of BCuNps. Cells, at 90 % confluence were treated with the BCuNps and native copper followed by incubation at respective temperature for 48 h. Cell viability and cytotoxic effect were assessed using MTT colorimetric assay. After 48 h, medium containing drugs was removed from each wells and wells were washed with 100 µL of phosphate buffer saline (PBS), pH-7.4. 50 µL of MTT reagent (2 mg/ml in PBS) was added in each well and plate was incubated for 4 h in CO₂ incubator. After 4 h, MTT solution was removed gently and formed formazan crystals were dissolved in 50 µL of DMSO by mixing and the absorbance of wells was read at 540 nm using microplate reader. Percentage cell death was calculated by formula,

\[
\text{Percentage cell death} = \left( \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \right) \times 100
\]

CTC₅₀ (concentration required to kill 50 % cells) of the test drugs were determined by plotting a graph between concentration and percentage cell death using Graph pad prism 5.

7.2.3. Wound healing studies

HaCat (Human skin keratinocytes) cells were used for assessment of wound-healing activity of BCuNps. The cells were cultured and maintained in DMEM and Hams F12 medium (1:1) supplemented with 10 % FBS.

7.2.3.1. Cell viability and proliferation assay (Denizot and Lang, 1986, Mosmann, 1983)

HaCat cells were seeded in 96 well plate at cell count of 8-10 × 10³ cells/well. Cells were treated with different concentrations of BCuNps and native copper in maintenance media before 90 % confluency and incubated for 24, 48 and 72 h. Cell viability at different concentrations of drug was measured by MTT (2 mg/ml) method and CTC₅₀ was determined. Cell proliferation assay was done at 50 % confluency of cells in 96 plate with BCuNps and native copper concentration below CTC₅₀ for 48 h. Cell viability and proliferation was assessed after estimating the cell death by MTT assay. Percentage cell viability as compare to control was calculated by following formula

\[
\text{Percentage cell viability} = 100 - \text{Percentage cell death}
\]
7.2.3.2. Cell migration assay/Scratch wound assay (Cory, 2011)

HaCat cells were seeded in 6 well plate at the cell count of $2 \times 10^5$ cells/well. At 95-100% confluence, a scratch was made in centre of each well of 6 well plate with sterile pipette tip and washed with PBS to remove scratched cells. Given scratched wound was photographed (0.0 h) immediately. These wounded cells were treated with BCuNps and native copper at 10 μg/mL concentration for 24 h in duplicate and remaining two wells were used as assay control. Cell migration in scratched wound was photographed again after 24 h to record cell migration in each well.

7.2.3.3. Anti-inflammatory effect on COX-2 expression (Chomczynski and Sacchi, 1987, Chomczynski and Sacchi, 2006, Raj et al., 2010)

HaCat Cells were seeded in 6 well plate at the cell count of $2 \times 10^5$ cells/well. At 90% confluency, cells were incubated with 1 mL of lipopolysaccharide (LPS) solution (10 μg/ml in serum free media) for 4 h in CO₂ atmosphere. After 4 h, LPS solution was removed and cells were washed with PBS. These cells were incubated with BCuNps and native copper at 10 μg/mL concentration for 6 h. After treatment, drug solution was removed and cells were treated with 1 mL of solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate of pH 7.0, 0.5% (w/v) N-lauroylsarcosine and 0.1 M 2-mercaptoethanol) for single step extraction of RNA. Briefly, cell lysate was re-suspended in solution D by multiple mixing with 1 mL micropipette and transferred in RNase free falcon tube. This cell lysate (1.0 ml) was mixed thoroughly by inversion with 0.1 mL of 2 M sodium acetate and 1.2 mL mixture of water saturated phenol, chloroform and isoamyl alcohol (25:24:1). This mixture was shaken vigorously on Vibramax 110 (Heidolph, Germany) for 2 min at 2000 rpm. Samples were cooled and centrifuged for 15 min at 12000 rpm and 4 °C in cooling centrifuge. Upper aqueous phase was collected carefully after centrifugation and incubated with 1.0 mL of isopropanol in new RNase free tube for overnight precipitation at (-) 20 °C. After incubation total RNA was pelleted by centrifugation for 30 min at 12000 rpm and 4 °C in cooling centrifuge. Supernatant was discard and RNA pellet was dissolved in molecular biology grade water. Amount and quality of RNA was estimated by BioPhotometer Plus (Eppendorf, Germany) and stored at (-) 20 °C until used.

COX-2 expression in drug treated, LPS treated and control cells was estimated at mRNA level by reverse transcriptase-polymerase chain reaction (RT-PCR) using QIAGEN One Step RT-PCR kit (Quagen, Germany) in Mastercycler Pro (Eppendorf, Germany). Primers for COX-2 amplification was procured from Integrated DNA Technologies (IDT, USA) with the sequence
F-5’GAGCCATACAGCAAATCC3’ and R-5’GGGAGTCGG GCAATCATC3’. Primer stock solution was prepared in molecular biology grade water. Reaction components were mixed according to manufacturer instructions with slight modification. Briefly, all the reaction components were thawed and mixed properly. The master mix was prepared separately in PCR tubes for isolated templates RNA according to Table 7.1. The master mix was maintained on ice until the start of thermal cycles for RT-PCR.

### Table 7.1. Components of the master mix for RT-PCR

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Name of component</th>
<th>Stock concentration</th>
<th>Volume/Reaction</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5X QIAGEN OneStep RT-PCR Buffer (MgCl₂)</td>
<td>12.5 mM</td>
<td>5.0 µL</td>
<td>2.5 mM</td>
</tr>
<tr>
<td>2</td>
<td>dNTP mix</td>
<td>10 mM of each</td>
<td>1.0 µL</td>
<td>400 µM of each</td>
</tr>
<tr>
<td>3</td>
<td>Primer F</td>
<td>30 µM</td>
<td>1.0 µL</td>
<td>1 µM</td>
</tr>
<tr>
<td>4</td>
<td>Primer R</td>
<td>30 µM</td>
<td>1.0 µL</td>
<td>1 µM</td>
</tr>
<tr>
<td>5</td>
<td>QIAGEN OneStep RT-PCR Enzyme mix</td>
<td>-</td>
<td>1.0 µL</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Template RNA</td>
<td>10 µg/mL</td>
<td>5.0 µL</td>
<td>50 ng</td>
</tr>
<tr>
<td>7</td>
<td>RNase-free water</td>
<td>-</td>
<td>11.0 µL</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td><strong>Total Volume</strong></td>
<td></td>
<td><strong>25 µL</strong></td>
<td></td>
</tr>
</tbody>
</table>

Mastercycler Pro was programmed for both thermal cycles, first, formation of cDNA of cox-2 mRNA by RT and second, amplification PCR cycle for COX-2 template cDNA (Table 7.2). RT-PCR programme was started and PCR tube with the master mix was transferred into the thermal cycler at 50 °C. After completion of cycle, PCR products were mixed with gel loading dye and analysed on 2% agarose gel containing 0.5 µg/ml ethidium bromide along with DNA ladder (100-10,000 base pair). Electrophoresis was then carried out with 1× TAE buffer at a constant voltage of 50 V for 1 h. Bands were visualised under a UV transilluminator.
### Table 7.2. Thermal cycler program for RT-PCR

<table>
<thead>
<tr>
<th>Step</th>
<th>Name of event</th>
<th>Duration</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1</td>
<td>Reverse transcription</td>
<td>30 min</td>
<td>50 °C</td>
</tr>
<tr>
<td>Step 2</td>
<td>Initial PCR activation step</td>
<td>15 min</td>
<td>95 °C</td>
</tr>
<tr>
<td>Step 3</td>
<td>Initial denaturation step</td>
<td>1.0 min</td>
<td>94 °C</td>
</tr>
<tr>
<td>Step 4</td>
<td>Denaturation</td>
<td>30 sec</td>
<td>94 °C</td>
</tr>
<tr>
<td></td>
<td>Annealing</td>
<td>45 sec</td>
<td>50 °C</td>
</tr>
<tr>
<td></td>
<td>Extension</td>
<td>1 min</td>
<td>72 °C</td>
</tr>
<tr>
<td></td>
<td>Repeat of Step 4 cycle</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>Step 5</td>
<td>Final extension</td>
<td>10 min</td>
<td>72 °C</td>
</tr>
<tr>
<td>Step 6</td>
<td>Storage</td>
<td>As required</td>
<td>4 °C</td>
</tr>
</tbody>
</table>

#### 7.2.4. Anticancer studies

During preliminary screening on various cancerous cell lines, BCuNps were found to be very effective against human lung adenocarcinoma epithelial cell line (A549). Therefore, anticancer studies of BCuNps were performed against A549 cell line. The cells were cultured and maintained in DMEM supplemented with 10% FBS. Cytotoxic effect was evaluated by MTT assay. Oxidative stress generated was measured by DCH-FDA fluorescence dye method. Genotoxic effect was studied by nuclear staining and flowcytometry analysis. Copper sulphate was used as control for all the studies.

#### 7.2.4.1. Cytotoxic effect of BCuNps (Tiwari et al., 2014)

Cytotoxic effect of BCuNps was determined by MTT assay. A549 cells were seeded in 96 well plate at cell count of 8-10 ×10^3 cells/well in 100 μL of proliferation media and incubated proliferation. Different concentrations for BCuNps and native copper were prepared in maintenance media. Cells were treated with different concentrations before 90 % confluence for 24 h and 48 h. After treatment, medium containing drug was removed from each well and wells were washed with 100 μL of PBS. Then, 50 μL of MTT reagent (2 mg/ml in PBS) was
added in each well and plate was incubated for 4 h in CO\textsubscript{2} incubator. After 4 h, MTT was removed and formed formazan crystals were dissolved in 50 µL of DMSO and plate absorbance was read at 540 nm using microplate reader. Percentage cell death was calculated by formula, 
\[
\text{Percentage cell death} = \left(\frac{A_c - A_t}{A_c}\right) \times 100,
\]
where \(A_c\) is absorbance of control and \(A_t\) is absorbance of test. CTC\textsubscript{50} of the test drugs were determined by plotting a graph between concentration and percentage cell death as compared to control using Graph pad prism 6.

7.2.4.2. Oxidative stress estimation (Reddy et al., 2015)

For the cytotoxic effect on A549 cell line, oxidative stress of BCuNps was determined by fluorescence based assay. Native copper and BCuNps were used at same concentration (3 µg/mL) to compare their effects. Cells were seeded in 6 well plate at the cell count of 2 \(\times\) 10\textsuperscript{5} cells/well. Before 90 % confluency, cells were treated with BCuNps and native copper at CTC\textsubscript{50} of each drug in maintenance medium and incubated for 24 h. After 24 h, cells were washed with PBS to remove any drug components. Cells were treated with 1 mL of 20 µM solution of DCHFDA (2’, 7’ –dichloro fluorescein diacetate) in each well and incubated for 30 min. These DCHFDA treated cells were collected from each well by scrapping with cell scrapper and centrifuged to get cell pellet. These cell pellets were suspended in PBS for fluorescence assay. The cellular fluorescence due to oxidative stress was analysed by flow cytometry using Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA) with excitation at 488 nm and emission at 575/540 nm. A minimum of 10,000 events was acquired and analysis of flow cytometric data was performed.

7.2.4.3. Genotoxic effect of BCuNps

Genotoxic effect of BCuNps was assessed by apoptotic activity, DNA fragmentation assay and Cell cycle analysis. BCuNps and native copper were used at equivalent concentration (3 µg/mL) for comparative effect on nuclear DNA.

7.2.4.3.1. Apoptotic activity of BCuNps (Chaudhary et al., 2015)

Apoptotic activity of BCuNps was analysed by nuclear staining using acridine orange stain. BCuNps and native copper solution was made in maintenance media below their CTC\textsubscript{50} value (2 µg/mL). A549 cells at cell count of 50 \(\times\) 10\textsuperscript{3} cells/ mL was seeded in 6 well plate. After attachment of the cells, they were treated with prepared drug concentration and incubated for 24 h. After 24 h, medium was removed from each well and cells were washed with PBS. Cell fixation was performed by 1 ml of 90 % ice cold methanol for 30 min at (-) 20 °C. After
fixation, methanol was removed and wells were washed three times with PBS. Nucleus of cells were stained with 500 µL of acridine orange stain in PBS (30 µg/mL) and incubated for 30 min in CO₂ incubator. After staining, unbound dye was washed with PBS. These cells were observed in fluorescence microscope and fluorescent images of cell were photographed apoptotic effect of drugs was analysed.

7.2.4.3.2. DNA fragmentation effect of BCuNps (Basnakian and James, 1994, Koganti et al., 2013)

Genotoxicity of BCuNps was analysed by DNA fragmentation effect. A549 cells were seeded in 6 well plate with cell count of 2 × 10⁵ cells/mL. After achieving monolayer, cells were treated with BCuNps and native copper at the concentration at 3 µg/mL, for 24 h. After the treatment, cells were trypsinized using trypsin-EDTA solution and cell pellet was obtained after centrifuging at 3000 rpm for 5 min at 4 °C. Supernatant was removed and the cell pellet was suspended with 1 mL digestion buffer (Tris, pH 7.5 10 mM, NaCl 100 mM, EDTA 1 mM and 1% SDS) and 50 µL of 50 mg/mL proteinase k was added followed by incubation at 45°C for 4 h. After incubation, equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) mixture was added, mixed, and centrifuged for 3000 rpm for 5 min at 4 °C. This step was repeated twice followed by a chloroform extraction. The aqueous phase was treated with 50 µl of RNase at 40 mg/ml at 37 °C for 2 h. Ice cold 70 % ethanol was added to the aqueous phase at twice volume to precipitate DNA in the presence of 10 % sodium acetate (0.3 M) and incubated for DNA precipitation over night at (-) 20 °C. The precipitated DNA was pelleted by centrifugation at 12,000 rpm for 20 min at 4 °C. The DNA pellet was re-suspended in 50 µL TE buffer (10 mM Tris HCl and 1 mM EDTA, pH 8.0) and quantified using UV spectrophotometer. 1 µg of DNA was subjected to 2 % agarose gel electrophoresis in 1× TAE (40 mM Tris HCl, 20 mM Acetate and 1 mM EDTA) buffer at a constant voltage of 30 V for 4 h. Bands were visualised under a UV trans-illuminator at the wavelength of 365 nm.

7.2.4.3.3. Cell cycle analysis (Chaudhary et al., 2015, Reddy et al., 2015)

Cell cycle analysis was conducted for understanding the cell phase distribution after treatment with BCuNps using propidium iodide stain. A549 cells were seeded in T 25 flask with cell count of 1 × 10⁵ cells/mL and incubated at 37 °C in 5 % CO₂atmosphere. After monolayer 50 % confluency, cells were treated with BCuNps and native copper containing equivalent amount (1 µg/mL) prepared in maintenance medium for 48 h. After treatment, cells were harvested using trypsin-EDTA solution, followed by centrifugation at 1500 rpm for 3 min at 4 °C. The
pellet was re-dispersed in PBS and centrifuged again to remove the culture medium. The cells were then fixed in 70% ice cold ethanol for 30 min at -20°C. Ethanol was removed after centrifugation at 1500 rpm for 3 min at 4°C and cells were suspended in 1mL of PI solution containing 25 µg/mL propidium iodide, 40 µg/mL RNAase and 0.03% Triton-X solution in PBS. The cells were analyzed using Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA) using excitation at 488 nm and emission at 575/540 nm. A minimum of 10,000 events was acquired and analysis of flow cytometry data was performed.

7.2.5. Bone turnover marker assay (Lee et al., 2004, Creeper et al., 2009, Cuong et al., 2009, Hapidin et al., 2015)

We evaluated the effect of BCuNps supplementation on bone turnover marker and on cell proliferation and differentiation using human foetal osteoblast cell line (hFOB 1.19-ATCC number: CRL-11372). Native copper was used to compare bone formation effect of copper in BCuNps. hFOB cells were cultured and proliferated in growth medium (DMEM and HamF12 at 1:1 ratio, with 0.3 mg/mL geneticin G418) at 33.5 °C and 5% CO₂ atmosphere. Cell differentiation studies were done with differentiation medium (growth medium with 50 µg/mL ascorbic acid and 5 mM β glycerophosphate) at 39 °C and 5% CO₂ atmosphere.

7.2.5.1. Cell viability assay (Hapidin et al., 2015)

Cell viability studies of BCuNps against hFOB cells was conducted to determine the CTC₅₀ value. hFOB cells were seeded in 24 well plate at cell count of 4-5 × 10⁴ cells/mL in 500 µL of growth medium for proliferation. Before 90% confluency, cells were treated with different concentrations of BCuNps and native copper in maintenance media and incubated at CO₂ atmosphere for 48 h. After 48, cell viability was estimated by MTT method.

7.2.5.2. Cell differentiation assay (Hapidin et al., 2015)

Cell differentiation of osteoblast cells is starting step of bone formation. hFOB cells were seeded in 6 well plate in growth medium at cell count of 4-5 × 10⁴ cells/mL and incubated at 33.5 °C for cell proliferation. BCuNps and native copper solution, below CTC₅₀ value (2 µg/mL), were prepared in differentiation media. After 80-90% confluency, these cells were incubated with drug solution and control media without any drug at 39 °C for differentiation for 1 day. Similarly, three 6 well plates were prepared and incubated for 3, 7, and 14 days. Differentiation media of each plate was changed in each two days. Differentiation of the hFOB cells were recorded for different treatment with different time interval.
7.2.5.3. Collagen formation assay (Tullberg-Reinert and Jundt, 1999, Cuong et al., 2009)

After cell differentiation of osteoblast cells, differentiated osteoblast cells starts collagen formation to form organic matrix of bone. Effect of BCuNps on collagen formation by hFOB cells was determined by Sirius Red stain based colorimetric assay. hFOB cells were seeded in 6 well plate in growth medium at cell count of 4-5 × 10^4 cells/mL and incubated at 33.5 °C for cell proliferation and after 90 % confluency, plate was incubated with BCuNps and native copper (2 µg/mL) in duplicate along with two control wells at 39 °C for 1 day. After 24 h, media containing drugs was removed and wells were washed with PBS to remove any trace of drug and medium. After this, cells were fixed with 1 mL of freshly prepared Bouins fluid which was prepared by mixing saturated aqueous picric acid solution (15 mL), 35% formaldehyde (5 mL) and glacial acetic acid. Cells were incubated with fixation solution for 1 h and after incubation, fixation liquid was removed using micro pipette gently followed by washing with running tap water for 30 min. After washing, plate was air dried and 1 ml of Sirius Red dye solution (1 mg/mL Sirius Red in saturated picric acid) was added in each well and incubated for 1 h on rocker. Thereafter, the dye solution was removed by gentle pipetting and cells were washed, 4 times with 0.01 N hydrochloric acid. Stained cells were photographed at 10 X magnification. After photo documentation, stain was dissolved with 500 µL of 0.1 N sodium hydroxide solution and absorbance of this solution was read at 540 nm using micro titre plate reader for collagen estimation. Similarly, hFOB cells were treated for 3, 7, and 14 days incubation with BCuNps and native copper in differentiation medium along with a control. Well contents were changed after each two days with respective drug solution. After completing the duration of incubation, cell were stained with Sirius Red and stained cells were photo documented followed by determination of total collagen. Images for 1, 3, 7 and 14 day treatment were analysed for collagen formation and the total collagen concentration were compared.

7.2.5.4. Calcium deposition assay (Dahl, 1952, Creeper et al., 2009, Cuong et al., 2009)

Formation of organic matrix by cross linked collagen fibrils helps in deposition of inorganic matrix of calcium phosphate. Effect of BCuNps on calcium deposition by differentiated hFOB cells was determined by Alizarin Red S stain based colorimetric assay. hFOB cells were seeded in 6 well plate in growth medium at cell count of 4-5 × 10^4 cells/mL and incubated at 33.5 °C for cell proliferation and after 90 % confluency, plate was incubated with BCuNps and native copper in duplicate (2 µg/mL) along with two control wells at 39 °C for 24 h . After 24 h, media containing drug was removed and wells were washed with PBS to remove any trace of drug.
and medium. After this, cells were fixed with 1 mL ice cold 90% ethanol for 1 h, at 4 °C. After 1 h, ethanol was removed and cells were stained with Alizarin Red S (40 mM) for 30 min. Thereafter, the dye solution was removed by gentle pipetting and cells were washed with water. Stained cells were photographed at 10 X magnification. After photo documentation, bound dye was quantified by dissolving the stain with 500 µL of 33% glacial acetic acid and absorbance of this solution was read at 540 nm using micro titre plate reader for calcium deposition. Similarly, hFOB cells were seeded for 3, 7, and 14 days incubation with BCuNps and native copper in differentiation media along with a control. Well content were changed after each two days with respective drug solution. After completing the duration of incubation, cell were stained with Alizarin Red S and stained cells were photo documented followed by determination of total calcium deposition. Images for 1, 3, 7 and 14 days treatment were analysed for collagen formation and the total collagen concentration were compared.

7.2.5.5. Alkaline phosphatase assay (Cuong et al., 2009, Lowry et al., 1951)

Alkaline phosphatase (ALP) is bone turn over marker for bone remodelling. Effect of BCuNps on alkaline phosphatase activity was estimated by alkaline phosphatase assay kit. hFOB cells were seeded in 6 well plate in growth medium at cell count of 4-5 x 10^4 cells/mL and incubated at 33.5 °C for cell proliferation and after 90% confluency, plate was incubated with BCuNps and native copper (2 µg/mL) in duplicate along with two control wells at 39 °C for 3 day. Plate was supplemented with fresh drug solution in differentiation medium. After 3 days’ treatment, cells were harvested using trysin-EDTA solution, followed by centrifugation at 3000 rpm for 3 min at 4 °C. Supernatant was removed and the cell pellets were washed with PBS to remove media and drug components followed by pelleting at 3000 rpm for 3 min at 4 °C. Cell pellets were suspended and incubated with 1 mL digestion buffer to lyse the cells. Total protein concentration in each cell lysate was estimated by Lowry method. ALP activity was estimated with ALP assay kit. Thereafter, enzyme activity per mg of total protein was calculated for BCuNps, native copper and control.
7.3. Results

7.3.1. Antimicrobial effects of BCuNps

Native copper which was used as control had shown good antimicrobial activity against all the tested organisms except E. coli. While BCuNps at the same concentration, had shown better antimicrobial activity even against E. coli where zone of inhibition had increased by more than two fold than the control (Table 7.3). While agar diffusion method is well accepted only for qualitative but not for quantitative antimicrobial studies but micro dilution assay is accepted for both. When tested using agar diffusion method, no significant difference in the antimicrobial activity was observed, between native copper and BCuNps, against P. aeruginosa. However, there was significant difference in micro dilution assay. BCuNps showed better antimicrobial effect and found to be highly effective against Gram positive bacteria viz., B. subtilis (6.25-3.125μg/ml) and S. aureus (50-25 μg/ml). B. subtilis was found to be more sensitive for BCuNps as compared to other organisms and comparable with the standard, ciprofloxacin.

Table 7.3. Antimicrobial activity of native copper and BCuNps

<table>
<thead>
<tr>
<th>Name of organism</th>
<th>Agar Diffusion Method (Zone diameter in mm)</th>
<th>Micro dilution assay using micro-dilution method for MIC (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Native copper (1mg/ml)</td>
<td>BCuNps (1mg/ml)</td>
</tr>
<tr>
<td>E. coli</td>
<td>8±1.0</td>
<td>18±0.5</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>34±0.6</td>
<td>37±0.4</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>30±0.8</td>
<td>37±0.6</td>
</tr>
<tr>
<td>S. aureus</td>
<td>23±0.8</td>
<td>34±0.5</td>
</tr>
</tbody>
</table>

x=results represent three individual experiments, y=results represent three individual experiments and CIPX=Ciprofloxacin

7.3.2. Cell viability and cytotoxicity effects of BCuNps

Cell viability assay studied using various normal cell lines confirmed that BCuNps are less toxic compared to native copper (Figure 7.1). Though, CTC50 for cancerous cells was comparatively less than normal cells, the results were inconsistent for different cancerous cell...
lines. BCuNps were found to be safer in case of MCF-7, CaCO-2 and HepG2 and cytotoxic for A549, Neuro 2a, HCT116 and Raw264.7 cell cultures (Figure 7.2).

![Cytotoxicity effect of native copper and BCuNps on normal cell lines.](image1)

**Figure 7.1.** Cytotoxicity effect of native copper and BCuNps on normal cell lines. Significant difference in cytotoxic effect of BCuNps as compared to native copper (*p<0.05, **p<0.01)

![Cytotoxic effect of native copper and BCuNps on cancer cell lines.](image2)

**Figure 7.2.** Cytotoxic effect of native copper and BCuNps on cancer cell lines. Significant difference in cytotoxic effect of BCuNps as compared to native copper (**p<0.01 and ***p<0.001)
7.3.3. Wound healing effects of BCuNps

The CTC$_{50}$ values of BCuNps was slightly higher, 31 $\mu$g/ml, when compared to native copper (28 $\mu$g/ml) (Figure 7.3). BCuNps enhanced the rate of cell proliferation as compared to native copper (Figure 7.4). BCuNps showed better cell migration in scratch wound assay (Figure 7.5) and healed the wound within 24 h (Figure 7.6). Table 7.4 represents the initial and final scratch width and % cell migration. RT-PCR studies of COX-2 expression showed that BCuNps exhibited better anti-inflammatory activity than native copper (Figure 7.7).

![Figure 7.3](image1.png)

**Figure 7.3. Cytotoxic effect of native copper and BCuNps on HaCat cells. Significant difference in cytotoxic effect of BCuNps as compared to native copper (**p<0.05 and ***p<0.01)**

![Figure 7.4](image2.png)

**Figure 7.4. Cell viability and Cell proliferation assay on HaCat cells**

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Figure 7. 5. Scratch wound assay on HaCat cells

Table 7. 4. Cell migration assay

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Scratch width at 0 h (n=8)</th>
<th>Scratch width at 24 h (n=8)</th>
<th>% Cell migration in 24 h (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>37.41±0.64</td>
<td>18.75±1.11</td>
<td>49.87±1.11</td>
</tr>
<tr>
<td>Native copper</td>
<td>33±1.16</td>
<td>13.15±3.08</td>
<td>60.16±3.08</td>
</tr>
<tr>
<td>BCuNps</td>
<td>32.23±0.75</td>
<td>2.01±3.05</td>
<td>93.76±3.05</td>
</tr>
</tbody>
</table>

Figure 7. 6. % cell migration in 24 h. Significant improvement in cell migration with BCuNps as compared to control (a=p<0.0001) and native copper (b=p<0.0001)
7.3.4. Anticancer effects of BCuNps

Cytotoxic concentration (CTC50), for A549 cells of BCuNps, was found to be 3.27 µg/mL after 48 h which was significantly higher than that of native copper (6.28 µg/mL) (Figure 7.8). Anticancer oxidative stress studies showed that BCuNps were more potent than native copper in generation of free radical for oxidative damage of cells (Figure 7.9). Acridine orange staining had shown that BCuNps had induced apoptotic pathway at tested concentration when native copper was not that much effective (Figure 7.10). DNA fragmentation pattern had shown that BCuNps have genotoxic effect on A549 cells (Figure 7.11). Cell cycle analysis showed S phase arrested of the cells was improved from 12.6 % by native copper to 15.2 % with BCuNps while control cells were having only 7.1 % cells in S phase (Figure 7.12).

![Figure 7.8. Cytotoxic effect of native copper and BCuNps on A549 cells. Significant difference in cytotoxic effect of BCuNps as compared to native copper (**p<0.01 and ***p<0.001)](image-url)
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Figure 7.9. Oxidative stress by BCuNps and native copper on A549 cells.

Figure 7.10. Genotoxic effect of BCuNps by apoptotic pathway on A549 cells. Red arrow shows apoptotic events in the drug treated A549 cells.

Figure 7.11. Genotoxic effect of BCuNps by DNA fragmentation of A549 cells.
7.3.5. Effect on bone turn over markers

CTC$_{50}$ value of BCuNps and native copper for 48 h, against hFOB cells, was found to be 17.6 µg/mL and 14.9 µg/mL respectively (Figure 7.13). BCuNps at concentration ≤ 10 µg/mL have shown cell proliferation effect while native copper was cytotoxic at this concentration. Both, BCuNps and native copper were found to have cell proliferative effect at concentration less than 2.5 µg/mL. Therefore, bone turnover marker assay was performed at 2 µg/mL of BCuNps and native copper.

![Figure 7.12. Genotoxic effect of BCuNps on cell cycle of A549 cells](image)

![Figure 7.13. Cell viability assay of native copper and BCuNps on hFOB cells](image)
hFOB cells were appeared to be flat, long and expanded cells after incubating with differentiation media. On day 1, BCuNps treated cells were elongated and dense, completely covered the well space and native copper treated cells were also elongated with little void space while control wells were having large void space and less dense than others. One day 3, BCuNps and copper native treated cells were started overlapping each other while control cells were still have some space to cover in the plate. Few cell differentiation clusters could be noticed in BCuNps and native treated cells. On day 7, cell differentiation was observed clearly in all the groups. No. of cell differentiation clusters were more in case of BCuNps than others. On Day 14, bigger mineraloid clusters of differentiated cells were observed for BCuNps and native copper as compared to control (Figure 7.14).

<table>
<thead>
<tr>
<th>Day</th>
<th>Control</th>
<th>Native copper</th>
<th>BCuNps</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td>Day 3</td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>Day 7</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
</tr>
<tr>
<td>Day 14</td>
<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
</tbody>
</table>

*Figure 7.14. Bone formation effect of BCuNps by enhancing cell differentiation in hFOB cells*

BCuNps showed effect on bone mineralization by improving the collagen formation in extracellular matrix of hFOB cells (Figure 7.15). Formation of collagen fibrils could be observed with Sirius Red stain on day 1 and day 3. Collagen fibril cluster were observed after incubating for 7 and 14 days. No. of collagen fibrils and clusters were more for BCuNps than
copper native and control. Total collagen in BCuNps treated hFOB cells was significantly improved than control (p<0.001) and native copper (p<0.001) on different days (Figure 7.16).

<table>
<thead>
<tr>
<th>Day</th>
<th>Control</th>
<th>Native copper</th>
<th>BCuNps</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
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<td><img src="image4.png" alt="Image" /></td>
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<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>Day 7</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
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<tr>
<td>Day 14</td>
<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
</tbody>
</table>

*Figure 7. 15. Bone formation effect of BCuNps by enhancing collagen formation in hFOB cells*

*Figure 7. 16. Estimation of collagen in BCuNps treated hFOB cells. BCuNps showed significant difference in collagen formation as compared to control (****p<0.001) and native copper (###p<0.001)*

Deposition of calcium phosphate crystals could be observed with Alizarin Red S stain. On day 1, only few crystals of calcium deposition were seen as compared to drug treated cells. Calcium deposition improved with incubation period. On day 14, complete mineralization were observed with BCuNps and native copper. Calcium deposition was better in BCuNps treated
differentiated hFOB cells (Figure 7.17). Mineralization of calcium phosphate crystal in BCuNps treated differentiated cells was higher than native copper and control (Figure 7.18).

![Bone formation effect of BCuNps by enhancing calcium deposition in hFOB cells](image1)

![Estimation of calcium deposition in BCuNps treated hFOB cells. BCuNps showed significant difference in calcium deposition as compared to control (\(*\star p<0.01, \(*\*\*p<0.001\) and native copper (\(*\#\#p<0.001\)))](image2)

ALP activity differentiated hFOB cells was improved with the treatment of BCuNps and native copper (Figure 7.19). ALP activity with BCuNps treatment was found to be 58.34 U/mg of protein which was significantly higher than control (\(p < 0.0001\)) and copper native (\(p < 0.001\)).
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Figure 7.19: Estimation of alkaline phosphatase activity in BCuNps treated cells after 3 days. BCuNps showed significant difference in alkaline phosphatase activity as compared to control (**p<0.001) and native copper (#p<0.001)

7.4. Discussion

Copper has been used for antimicrobial activity since ancient time (Borkow and Gabbay, 2009). It has been reported that copper had higher antimicrobial effect against Gram positive bacteria than Gram negative bacteria (Ruparelia et al., 2008). Moreover, CuNps had shown better antimicrobial activity than conventional form (Hajipour et al., 2012, Yoon et al., 2007). Various mechanisms had been reported for antibacterial activity of CuNps such as dissipation of cell membrane potential, generation of reactive oxygen species, lipid peroxidation, protein oxidation and DNA degradation etc (Chatterjee et al., 2014). Better antimicrobial activity of BCuNps over copper sulphate would definitely help to develop a biocompatible formulation of copper nanoparticles for their application in therapeutics.

Copper is an essential element for cell biology and every cell has a mechanism to deal with its load (de Romaña et al., 2011). CuNps had been reported for their cytotoxic effect on renal (Liao and Liu, 2012) and hepatic cells (Seth et al., 2004). However, these studies used CuNps which were synthesized by physical or chemical methods and had been performed with the aim to study the toxic effects of CuNps for accidental exposure to biologically incompatible CuNps. In an in vivo comparison study, copper ion, CuNps and copper macro particles were studied and copper ions were found to be most lethal than CuNps (Chen et al., 2006). Cytotoxic effect of CuNps is due to release of copper ions from them. In living cell, different copper transporter proteins have been reported which are known as copper chaperons (Nevitt et al., 2012). These copper chaperons help in transportation of copper in the body and maintain its homeostasis.
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The proteins produced by copper resistant organisms and the higher eukaryotes belong to same family of protein (Dupont et al., 2011).

For human, most important member of this family is ceruloplasmin, which is found in blood. It controls the toxic effect of free copper in our body and helps to transport copper in different tissues (Harvey and McArdle, 2008). BCuNps used in this study were coated with proteins excreted by copper resistant strain of Bacillus cereus. Therefore, Similarity of copper resistant proteins with ceruloplasmin would help to reduce the harmful effects of copper in BCuNps and would make them biocompatible. We had found that BCuNps were less toxic than native copper (CuSO$_4$) against normal cells (HaCat, Vero and hFOB). However cytotoxic effects of BCuNps against cancer cells were found to be inconsistent. BCuNps were safer than native copper in MCF 7, CaCO 2 and HepG 2 while they were found to be toxic for A549, Neuro 2a, HCT116 and Raw264.7. Enhancement in safety level, against these cell lines, could be a signature for biocompatibility of BCuNps and would prove as a better form of copper than conventional form, for supplementing in its physiological deficiency. CuNps had reported for dose dependent effect on COX-2 expression in macrophage cells (Arancibia et al., 2016). BCuNps showed significantly ($p<0.001$) higher cytotoxic effect against lung, colon and brain cancer cells. CuNps have been reported for anticancer effects earlier (Ahamed et al., 2010).

Copper has been found to be effective in the treatment of wound (Borkow et al., 2010b, Somayaji et al., 1995, Mulder et al., 1994). Copper works through different molecular mechanisms (Borkow et al., 2010a, Sen et al., 2002) and by affecting physicochemical parameters (Rakhmetova et al., 2010). It was reported to improve cell proliferation and anti-inflammatory cytokines which helps in wound healing (Philips et al., 2010). Copper peptides were very effective in the treatment of various types of wound (Parker et al., 2013, Konerding et al., 2012, Mulder et al., 1994). BCuNps were found to have faster healing in excision wound model (Tiwari et al., 2014). BCuNps, coated with proteins, at concentration below 10 µg/mL had improved proliferation of HaCat cells. Cell migration was found to be two times faster with BCuNps than control cells. BCuNps had shown effective suppression of LPS induced COX-2 expression, an inflammatory marker.

BCuNps were found to be highly potent against human lung carcinoma cells. They showed cytotoxic effect by generation of reactive oxygen species, formation of apoptotic bodies, fragmenting DNA and arresting the cells in S phase. Copper oxide nanoparticles have been reported to induce oxidative stress. CuNps have been reported to act as antitumor agent in dose
dependent manner by apoptotic pathway (Jose et al., 2011). Concentration of BCuNps required for anticancer effect was much lesser than its cytotoxic effect on tested normal cell lines which makes it safer for normal tissues. Copper nanoparticles were found to be more effective in damaging DNA by oxidative stress in lung cells by releasing copper ion in the cell culture medium. Rate and released amount of copper into the medium was reported to be higher with the CuNps than the macro particles (Midander et al., 2009).

Copper plays a specific role in angiogenesis and bone formation in dose dependent manner (Milkovic et al., 2014). Copper has been found to be effective in cell proliferation (Fiedler et al., 2011). In an earlier report, copper ions (0.056, 0.56 and 5.65 µg/cm²) were tested for in vitro bone formation in scaffold and were found to enhance the activity and proliferation of osteoblast cells (Ewald et al., 2012). Yang et al., 2010, has reported no effect of copper on cell differentiation of osteoblast, as the concentration tested were 650 ng/mL, 65 ng/mL and 6.5 ng/mL which were significantly less than the effective copper concentration (Yang et al., 2010). In another study, copper was reported to act in concentration dependent manner when studied with 5-50 µM (0.325-3.25 µg/mL) of copper and it was found that copper had modified both proliferation and differentiation of mesenchymal cells obtained from postmenopausal women (Rodríguez et al., 2002). Copper has been used as doped material on bioactive glasses and glass cremains for bone tissue engineering, osteo-genesis and angiogenesis (Hoppe et al., 2011). CuNps had shown significant effect on bone regeneration in bone tissue engineering (Dhivya et al., 2015). Biocompatible colloidal CuNps and native copper were studied for angiogenesis, cell proliferation at systemic and molecular level using chick embryo model and CuNps were found to be effective more than native copper in concentration dependent manner for pro-angiogenic and pro-proliferative properties (Mroczek-Sosnowska et al., 2015).

In this study, we found that BCuNps at lower concentration (BCuNps ≤ 10 µg/mL) had proliferative effect on hFOB cells. Cells differentiation was comparatively higher with BCuNps which could be due to its nano size. Therefore, they showed better effect in bone forming matrix than native copper. Copper resistant proteins present along with BCuNps could act as copper carrier proteins and help in transporting copper to copper binding enzymes like lysyl oxidase, superoxide dismutase (Dupont et al., 2011). Bone turnover marker like cell viability, cell differentiation, alkaline phosphatase activity, collagen content and calcium deposition improved with the treatment of BCuNps and comparable with earlier reported with a synthetic drug for anti-osteoporosis effect (Cuong et al., 2010). Cell differentiation, collagen content and calcium deposition improved with incubation period significantly. Alkaline
phosphatase activity of osteoblast cells after 3 days of treatment had improved significantly (p <0.001) with BCuNps as found with an anti-rheumatic drugs (Kuriyama et al., 2002) and with a copper rich plant sourced calcium product (Adluri et al., 2010). Cell differentiation effect and alkaline phosphatase activity of hFOB 1.19 cells had improved with BCuNps treatment as discussed in earlier report with the treatment of mineral rich plant extract (Hapidin et al., 2015).

7.5. Conclusion

In the present study, we evaluated BCuNps produced by copper resistant soil isolate Bacillus cereus SWSD1 for antimicrobial activity, biocompatibility, wound healing effect, anticancer activity and bone formation potential. BCuNps were found to be effective antimicrobial agent and most effective against Gram positive bacteria. BCuNps had shown less toxic effect on normal cells. However, cytotoxic effects of BCuNps were inconsistent for cancer cells. They were found to be highly safe for normal kidney cells (Vero cells) and highly toxic for lung cancer cells (A549). BCuNps had shown promising effect on cell proliferation, cell migration and anti-inflammatory effects for their wound healing properties Anti-proliferative effect of BCuNps for lung cancer cells was evaluated by oxidative and genotoxic studies. BCuNps had shown concentration dependent anticancer effect and was effective at ~2 µg/mL concentration by inducing apoptosis, DNA fragmentation and cell cycle arrest. Bone turn over marker assays had shown potential role of BCuNps in bone formation and bone remodelling. BCuNps had shown improvement in cell differentiation and bone mineralization with hFOB 1.19 cells. These BCuNps could further evaluated for their effect at molecular level and in vivo effects for development of a biocompatible pharmaceutical product of copper.

7.6. References


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