Copper Nanoparticles Induce Apoptosis and Oxidative Stress in SW-480 Human Colon Cancer Cell Line

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Research Article

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Abstract

Cu nanoparticles (CuNPs) have various applications in biomedicine, owing to their unique properties. As the effect of CuNPs on the induction of oxidative stress and apoptosis in the human colorectal cancer cell line SW480 has not yet been studied, we investigated the toxicity and mechanism of action of these NPs in SW480 cells. An MTT assay was performed to assess the effect of the particles on the viability of SW480 cells. The levels of oxidative stress were assessed after 24h of treatment with CuNPs by evaluating the ROS production. Antioxidant enzyme activity was assessed using a colorimetric method. To investigate the effect of NPs on cellular apoptosis, Hoechst33258 staining was performed, and the expression of Bax, Bcl2, and p53 genes was evaluated by qRT-PCR. MTT assay results showed that CuNPs inhibited the viability of SW480 cells. Moreover, the increase in ROS production at all three concentrations (31, 68, and 100 µg/ml) was statistically significant. It has been observed that CuNPs lead to increased expression of Bax and p53, and decreased expression of Bcl-2. Hoechst staining was performed to confirm apoptosis. In conclusion, the induction of apoptosis demonstrated the anticancer potential of CuNPs.

Introduction

Cancer is responsible for the annual mortality of many patients, particularly those in developed countries(1). Typical cancer treatments, such as chemotherapy, have many side effects, and in addition to cancer cells, they can affect normal cells that have a high dividing power. Therefore, cancer treatment requires targeted therapy that has improved efficacy and fewer adverse effects (2, 3).

Colorectal cancer (CRC) is the second most lethal cancer in both male and female(4). Epidemiological studies have successfully identified many environmental factors, such as lifestyle, diet, anthropometrics, and pharmacological factors, associated with CRC risk. The identified risk factors include a family history of CRC, inflammatory bowel disease, cigarette smoking, physical activity, BMI, consumption of processed and red meat, alcohol consumption, height, obesity, and diabetes (5, 6). Some factors reduce the risk of CRC like, Physical activity(7), use of aspirin and nonsteroidal anti-inflammatory drugs (NSAIDs)(8) and postmenopausal hormone therapy(9).

Nanotechnology is an extensively studied field. Rapid growth in nanotechnology has led to the development of cancer treatments (10). Nanoparticles are 1–100 nm (11). These materials exhibit properties, such as submicron size, high surface-to-volume ratios, targeting rates, and dissimilarities (12). Contact between nanomaterials and cell lines can lead to a better response in cancer cells and can reduce the concentration of the desired compound(13). Over the last few years, metal nanoparticles have been used for pharmaceutical purposes. Moreover to their various applications, metal nanoparticles can produce reactive oxygen species, which can cause damage to and death of cancer cells (14). ROS formed by nanoparticles can damage DNA/RNA, membrane walls, and organelles, eventually leading to necrosis and apoptosis(15). The body contains various enzymatic antioxidants that prevent oxidative stress,

including superoxide dismutase (SOD), catalase, and peroxidase(16). An oxidant/oxidant imbalance leads to "oxidative stress"(17).

Bax is a member of the Bcl-2 family that causes apoptosis. BAX gene expression is enhanced by the p53 tumor suppressor gene(18). The Bcl2 family of proteins is anti-apoptotic and plays an important role in mitochondrial apoptosis. Stimulants of apoptosis can reduce the protein expression in this family(19). Cu is an important mineral that plays a vital role in the human body. The role of copper as a co-factor in some important enzymatic systems such as oxidoreductases has been identified and copper-containing proteins have several roles in electron and oxygen transport (20, 21). Cu nanoparticles (CuNPs) are highly practical owing to their unique properties. Several studies have shown that Cu-based nanoparticles can induce apoptosis via ROS overproduction and DNA injury(22). This study aimed to explore the anticancer effects of CuNPs on SW480 cells by examining the molecular mechanisms associated with apoptosis.

Materials And Methods

CuNPs of 99.9% purity were purchased from NANOSANY (Mashhad, Iran). The purchased CuNPs were 40 nm in size and were spherical. Figure 1 shows the TEM micrographs of the CuNPs.

Cell culture

The SW480 human colorectal adenocarcinoma cell line was purchased from the Pasteur Institute (Tehran, Iran) and maintained in RPMI-1640 (KRT100) medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The cells were incubated at 37°C in 5% CO2 and 95% air. Cells were exposed to 0.25% trypsin-EDTA, and those harvested between passages 2 and 10 were used in all experiments.

Determination of SW480 Cells Viability using MTT Assay

To assess the effect of the particles on the viability of the SW480 cell line, an MTT assay was performed, which is based on the reduction of MTT to a purple formazan product by mitochondrial dehydrogenase in intact cells. SW480 cells in the logarithmic growth phase were seeded in a 96-well plate at 1.1×10^4 cells/well and incubated overnight(23). After 24 h of incubation, to ensure the adhesion of cells to the plate bed, diluted CuNPs in RPMI with different concentrations (0, 4, 7, 200, 15, 31, 62, 100, and 125 µg/ml) (24) were added to each well in triplicate. After 24 h, the MTT reagent (5 mg/ml in PBS) was added to each well, and the cells were incubated for 4 h. The contents of the wells were carefully drained and replaced with 100 µl of dimethyl sulfoxide (DMSO). After 30 min of incubation, to ensure that the paint particles were dissolved, the light absorption of the wells at 570 nm was read using an ELISA plate reader (RT-2100C Microplate Reader, China). The viability of the SW480 cells was calculated using the following formula: (OD of CuNP-treated cells)/ (OD of untreated cells) × 100. The 50% inhibitory concentration (IC50) was calculated using GraphPad Prism 9 software.

Intracellular ROS measurement

ROS release was determined using a microplate fluorometric assay, according to the manufacturer's instructions (Kiazist, Iran). Briefly, SW480 cells were cultured in a 96-well black plate at a density of 22×10^3 cells per well. The next day, cells were treated with various concentrations of CuNPs (0, 31, 68, and 85 µg/ml). After 24-hour incubation, the medium was removed and the cells were exposed to DCFDA (2', 7'- dichlorofluorescein diacetate) solution (100 µl). The cells were incubated for 45 min at 37°C. Fluorescence intensity was measured at Ex/Em=485/528 nm (excitation/emission) using a fluorescence microplate reader (Bio-Tek Instruments, Winooski, USA).

Apoptosis assay by Hoechst 33258

Hoechst 33258, a DNA-specific fluorescent dye, was used to assess the nuclear morphology during apoptosis. The SW480 cells were seeded in a 6-well plate After 24 h they were treated with defined concentrations (0, 31, 68, and 85) of CuNPs and were incubated overnight. The samples were fixed with methanol for 15 min. Cells were stained with Hoechst 33258 at 37°C for 30 min. The stained cells were visualized under a fluorescence microscope (BEL, Italy).

RNA extraction and cDNA synthesis

Total RNA from treated and untreated SW480 cells was isolated using RNX-plus™ according to the manufacturer's protocol. The quantity and purity of the extracted RNAs were checked using a NanoDrop One UV–Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and %1 gel agarose electrophoresis. cDNA was synthesized using the Pars Tous Company Kit (Iran), according to the manufacturer's instructions. qRT-PCR was conducted using Syber Green qPCR Master Mix (Amplicon, Denmark), forward and reverse primers (Table 1), cDNA template, and RNase-free dH2O on a LightCycler 96 System (Roche, Germany). All gene sequences were designed using the Primer3 software. β-actin was used as an internal housekeeping gene. The expression levels of Bax, Bcl-2, and p53 were calculated based on the cycle threshold (Ct) number and $2^{-\Delta\Delta Ct}$ method.

Table 1. The sequences of the primers used in the RT-PCR

Gene	Forward	Reverse
Bcl-2	GAGCGTCAACCGGGAGATGT	TACAGTTCCACAAAGGCATCCCAG
Bax	GGT TGT CGC CCT TTT CTA CTT	GGA GGA AGT CCA ATG TCC AG
P53	TGTGACTTGCACGTACTCCC	ACCATCGCTATCTGAGCAGC
β-actin	TAA CGC AAC TAA GTC ATA	AAG ATC AAG ATC ATT GCT

Antioxidant markers measurement

CAT (Kiazist, KCAT-96, Hamedan, Iran), SOD (Kiazist, KSOD-96, Hamedan, Iran), and GPx (Kiazist, KGPx-96, Hamedan, Iran) activities were evaluated according to the manufacturer's protocol. SOD activity was

measured using a colorimetric method, in which resazurin (blue to purple) in the presence of O_2^- was converted to resorufine and changed to pink. The GPX kit uses a coupling reaction between the enzyme glutathione reductase and its coenzyme NADPH. CAT enzyme has peroxidase activity in the presence of methanol, but stops in the presence of its inhibitor, and its formaldehyde reacts with purpald to produce a purple color. The dye absorbed light at a wavelength of 540 nm. In the total antioxidant capacity (TAC) experiment, Cu^{+2} was reduced to Cu^{1+} in the presence of antioxidants and produced dye in the presence of a chromogen. In the total oxidant status (TOS) assay, ferrous metal is oxidized to ferric metal in the presence of oxidants and produces color in the presence of a chromogen.

Statistical Analysis

Data are expressed as mean ± standard deviation of triplicate experiments (n=3). Statistical analyses were performed using GraphPad Prism version 9. The MTT and enzymatic assay data were analyzed using one-way ANOVA followed by Tukey's test for the comparison of means among the different groups. Statistical significance was set at $P < 0.05$.

Results

The cytotoxicity of the CuNPs

The cytotoxicity of CuNPs against SW480 cells was measured using the MTT method at different concentrations (0, 4, 7, 15, 31, 62, 100, 125, and 200). The effect of Cu NPs on SW480 cell viability was analyzed after 24 h of incubation. The cell viability percentage at concentration 4 was not significant compared with that of the control cells. However, the other concentrations significantly decreased cell viability in a dose-dependent manner. The CuNPs in SW480 have an IC50 value of 68 µg/ml. Most cells were killed with 200 µg/ml CuNPs (Figure 2).

Determination of intracellular ROS

The capacity of CuNPs to generate ROS was studied using the DCFH-DA assay. As shown in Figure 3, intracellular ROS production after 24 h in SW480 cells was observed at a CuNP concentration of 31 µg/ml ($p < 0.05$). However, increase was statistically significant at 68 µg/ml and 100 µg/ml CuNPs compared with the control group (untreated cells) ($p < 0.001$).

Hoechst 33258 staining assay

Hoechst 33258 stains DNA and is commonly used to visualize nuclei and mitochondria. Nuclear density and fragmentation of DNA in apoptotic cells were detected using a fluorescence microscope (Figure 4). In this study, the effect of the desired concentrations of CuNPs after 24h on the rate of apoptosis in SW480 cells showed that CuNPs increased apoptosis in SW480 cells compared with that in the control group.

Antioxidant markers

The activities of the antioxidant enzymes CAT, GPX, and SOD in SW480 cells were measured after 24 h of treatment with CuNPs. As shown in Figure 5, CuNPs increased CAT activity at 68 µg/ml ($p < 0.05$) and 100 µg/ml ($p < 0.01$); however, no significant change was observed at 31 µg/ml. GPX enzyme activity after treatment with CuNPs showed a remarkable increase at 100 µg/ml ($p < 0.001$); however, no significant changes were observed at CuNP concentrations of 31 and 68 µg/ml. CuNPs also significantly increased SOD activity at concentrations of 68 ($p < 0.01$) and 100 µg/ml ($p < 0.001$). As shown in Figure 6, TAC at concentrations of 68 and 100 µg/ml CuNPs was significantly reduced ($p < 0.001$).

The mRNA expression of Bax, p53 and Bcl2 analysis

Bax, p53, and Bcl-2 mRNA expression levels were analyzed in SW480 cells treated with the desired concentrations of CuNPs for 24h. As shown in Figure 7, the mRNA expression of Bax in SW480 cells treated with 68 µg/ml ($P < 0.05$) and 100 µg/ml ($P < 0.001$) significantly increased, whereas at 31 µg/ml, there were no significant changes. The mRNA expression of p53 was remarkably increased at all tested concentrations, including 31 µg/ml ($p < 0.05$), 68 µg/ml, and 100 µg/ml ($p < 0.01$). On the other hand, Bcl2 mRNA expression after 24h of treatment with CuNPs significantly increased at concentrations of 68 µg/ml ($p < 0.01$) and 100 µg/ml ($p < 0.001$).

Discussion

Colorectal cancer (CRC) is a perilous disease in developed countries, with limited treatment options. Therefore, efforts are being made to develop new approaches to scrutinize and treat this disease(25).

The most important application of nanotechnology is treating diseases and improving health in a new way. Nanoparticles have a variety of nanoscale sizes with specific chemical and physical properties. Small nanoparticles can be absorbed by cells, causing cell toxicity (26, 27).

This study demonstrates the cytotoxicity of CuNPs in a colorectal adenocarcinoma cell line (SW480) and the possible mechanisms related to these NPs. Our results showed that by increasing the dose of CuNPs, the viability of cells decreased, with an IC50 of 68 µg/ml. In addition, the present findings showed that SW480 cells exposed to CuNPs exhibited increased ROS production. Oxidative stress, caused by ROS overproduction, can induce apoptosis. Following treatment with CuNPs, qRT-PCR analysis revealed that Bcl2 gene expression was reduced, whereas p53 and Bax gene expressions were increased. Furthermore, apoptosis induced by CuNPs was analyzed using Hoechst33258 staining and apoptotic cells were observed under a fluorescence microscope.

Previous studies have shown that CuNPs exert cytotoxic effects and induce apoptosis by increasing cellular oxidative stress (28–31) which is consistent with the results of the present study.

According to Saranya et al. (32), CuNPs inhibited the growth of MCF7 cells, and the IC50 value based on the MTT assay was 250 µg/ml. Mehdizadeh et al. evaluated the anticancer properties of CuNPs in K562 cells, in which the IC50 value was reported as 25.24 µg/ml (33). Furthermore, Sharma Purnima et al.

investigated the induction of oxidative stress by CuNP sizes of 11–14 nm, and MTT assay results showed that these NPs induced cytotoxicity in RAW 246.7 macrophage cells through oxidative injury(34). However, the IC50 of the synthesized green CuNPs against the HepG2 cancer cell line was significantly higher (IC50 = 500 µg/ml)(35). In addition, Shilapa et al. investigated the toxicity of biologically synthesized CuNPs (5–20 nm) in SK-MEL-3 cells. According to the MTT test results, after 48 h of incubation with 16 µg/ml CuNPs, SK-MEL-3 cell proliferation was significantly reduced(36). Song et al. studied the cytotoxicity of CuNPs with four different sizes (25, 50, 78, and 100 nm). Four different cell lines from two species were selected: mammalian (H4IIE, HepG2) and piscine (PLHC-1, RTH-149). The results showed that the piscine cell lines were more resistant than mammalian cell lines. In addition, the IC50 of NPs of selected sizes was significantly different; therefore, 25 nm CuNPs had the highest cytotoxicity in all cell lines(37). Moreover, the green synthesized CuNPs in the sizes of 39.09 to 18.9 nm showed the cytotoxicity effects on MCF7 cells and T3T normal cells, and IC50 values were reported in concentrations of 37.02 and 262 µg/ml, respectively. In addition, the AO/EtBr assay showed that CuNPs induced apoptosis in MCF7 cells(38). The toxicity of CuNPs synthesized from Quisqualis indica plant extract was evaluated using MTT and LDH assays in B16F10 melanoma cells and NiH3T3 (embryonic fibroblast cells). The IC50 value for B16F10 cells was reported to be 102 µg/ml, whereas toxicity was not high in normal cells. In contrast, the amount of intracellular ROS in treated B16F10 cells increased in a dose-dependent manner, and Annexin V-FITC staining showed that apoptotic damage was inflicted on these cells(24). In accordance with our results, the cytotoxicity of the green synthesized CuNPs in A2780-CP cells showed that ROS production caused apoptosis in A2780-CP cells. However, they are not toxic to normal foreskin fibroblasts (39). According to these studies, it seems different IC50s have been reported based on the type of synthesis, particle size, and different cell lines. In addition, evidence suggests that nanoparticles may induce cellular apoptosis by targeting the mitochondrial pathway, reducing Bcl-2 protein expression, increasing Bax and p53 expression, and caspase3 activation (40–42). Similar to our results, some studies have shown that treatment with CuNPs alters the expression of Bax, p53, and Bcl2 gene expression (43, 44). Consistent with our data, previous studies have shown that treatment with NPs can induce oxidative stress and increase antioxidant enzyme activity to maintain a redox state(45, 46).

ROS are produced in small amounts during physiological cellular activity. Oxidative stress via ROS overproduction can damage biological macromolecules such as DNA, lipids, and proteins, eventually causing apoptosis(47). Antioxidants can minimize the cytotoxic effects of ROS(48). Increasing antioxidant enzyme activity reduces the amount of ROS and maintains it at nontoxic levels.

Conclusion

Our results showed that exposure to CuNPs caused cytotoxicity and ROS-mediated apoptosis in SW480 colorectal adenocarcinoma. The increased expression of Bax and p53 and reduced expression of Bcl2 confirmed that CuNPs induced apoptosis pathway in sw480 cells. In addition, the activities of the antioxidant enzymes GPX, SOD, and CAT were increased; however, they could not completely counteract the overproduction of ROS.

Declarations

Acknowledgment

Authors' Contribution:

P.Gh. conducted and analyzed laboratory tests. N.Z. interpreted the data. P.Gh. wrote the manuscript. R.A. and Gh.Sh. were the project supervisors. All authors have read and approved the final manuscript.

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Data availability

All data used and analyzed during the current study are included in this manuscript and are available from the corresponding author upon reasonable request.

Compliance with ethical standards

Conflict of interest

There is no competing interest.

Ethics approval and consent to participate

This article does not contain any studies involving human participants or animals performed by any of the authors. The experimental procedure was approved by the Faculty of Medicine at the Hamadan University of Medical Sciences (IR.UMSHA > REC.1399.929).

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Figures

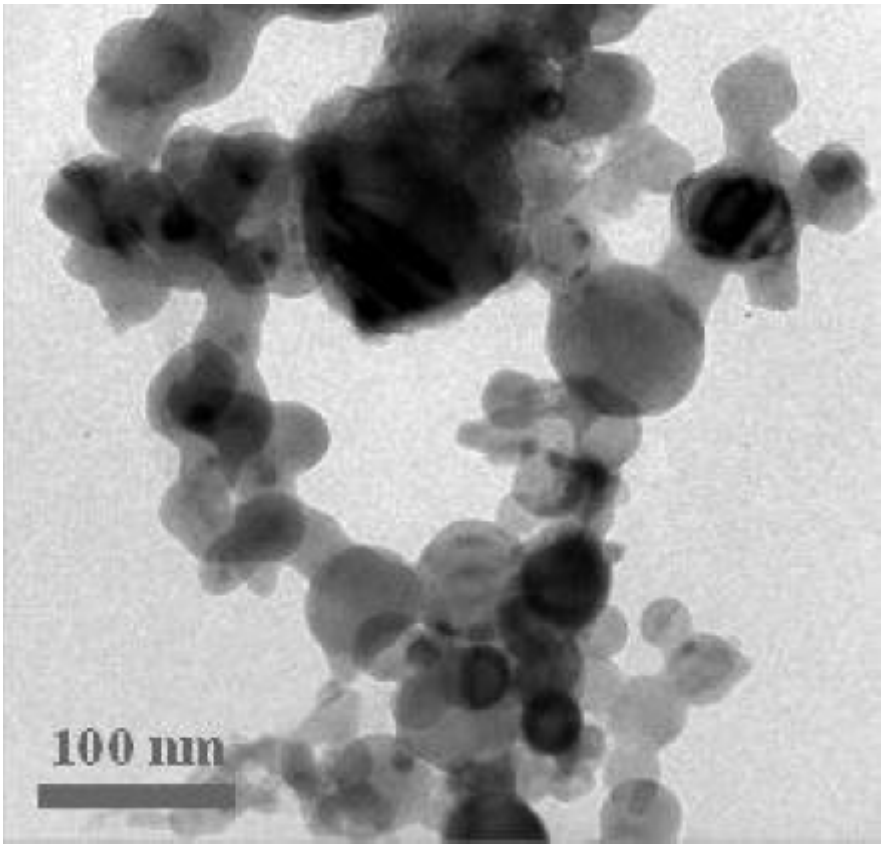

Figure 1

Transmittion Electron Microscope of Cu nanoparticles

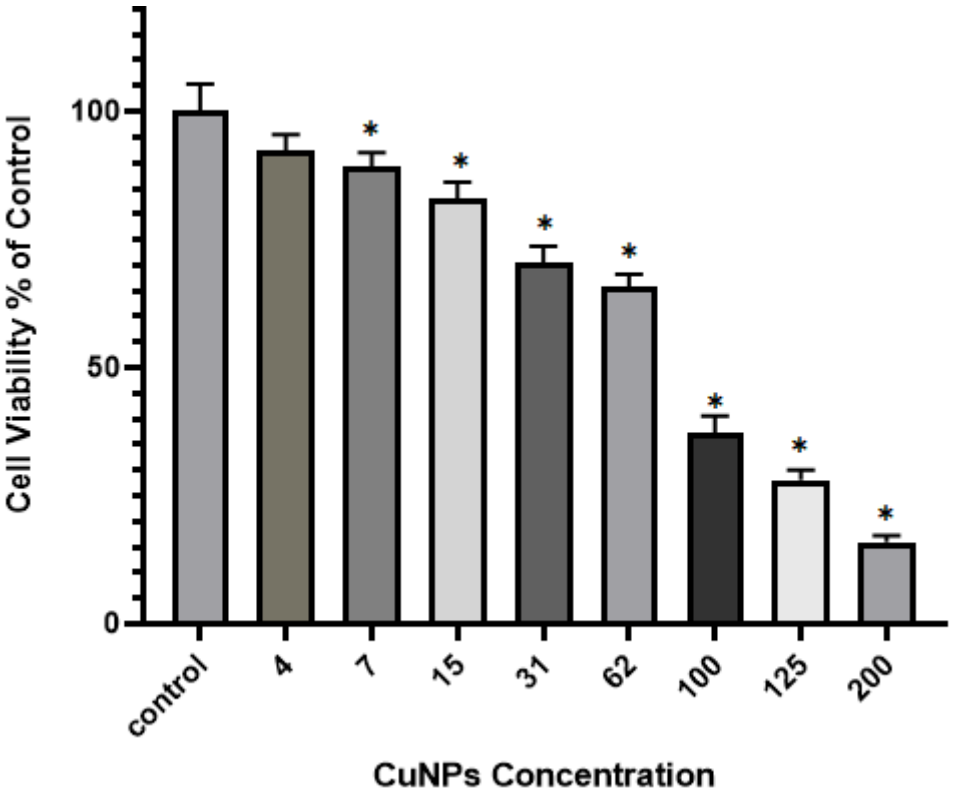

Figure 2

Cell viability curve after 24 hours CuNPs treatments on SW480 colorectal cancer cell line MTT Assay. Data are presented as a percentage of living cells relative to control cells. Increasing Increasing the CuNPs concentration reduces the viability of SW480 cells. * $p < 0.05$ comparedto control cells.

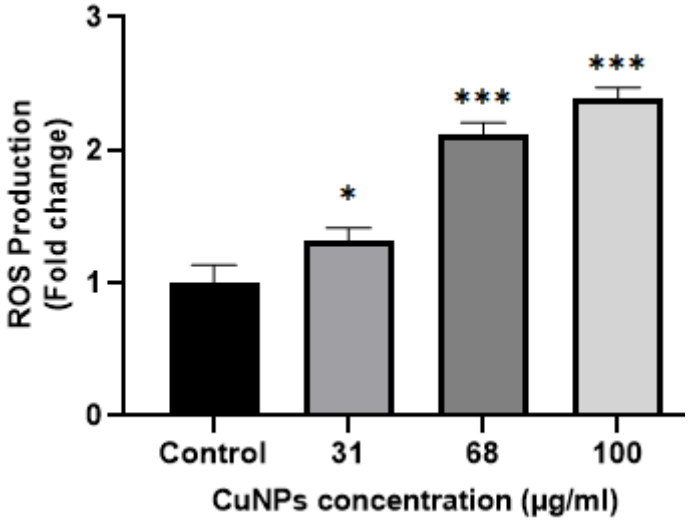

Figure 3

The effect of CuNPs on ROS production in SW480 colorectal cancer cells after 24 hours of treatment. Data were reported as mean ± SD in three replications (n = 3). * $p < 0.05$ and *** $p < 0.001$ compared to control cells.

Figure 4

Morphology of condensed chromatin and nuclear fragmentations after 24 hours of treatment with CuNPs on SW480 cells by Hoechst 33258 staining. A. Control cells (untreated SW480 cell line). B. Treated cells with 31 µg/ml of CuNPs. C. Treated cells with 68 µg/ml of CuNPs. D. Treated cells with 100 µg/ml of CuNPs.

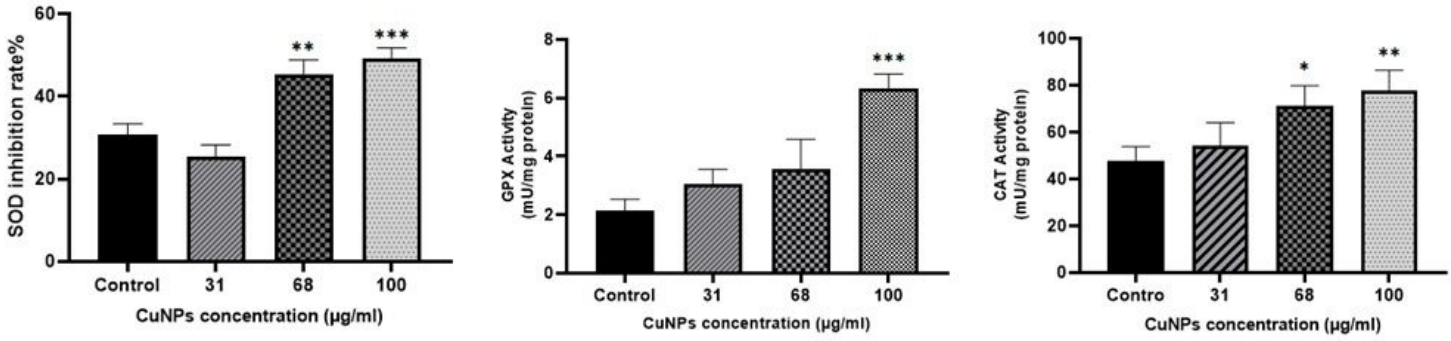

Figure 5

Effect of CuNPs on antioxidant enzyme activity in SW480 cells after 24h of treatment. * $p < 0.05$ ** $p < 0.01$ and ***$p < 0.001$. Data are expressed as mean ± SD(n=3).

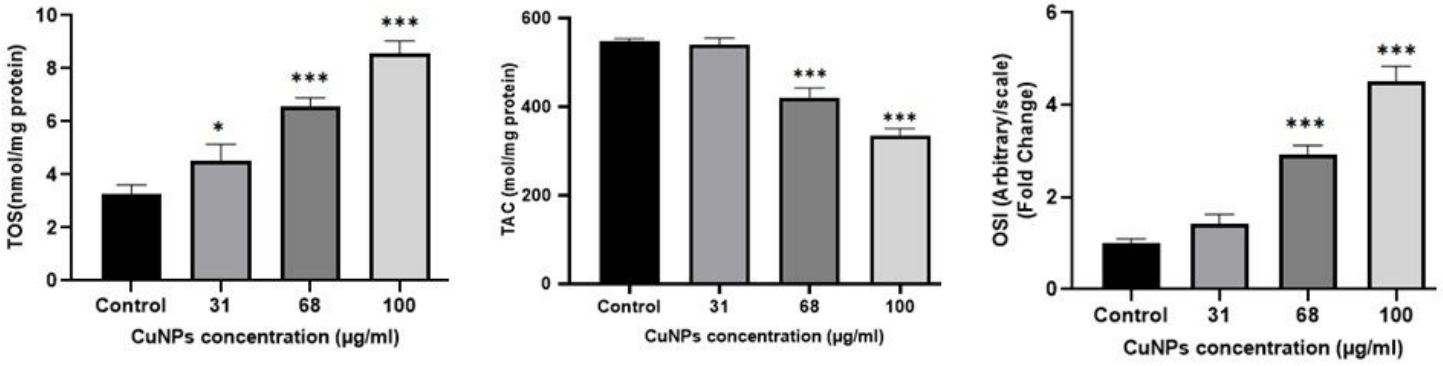

Figure 6

The findings of oxidative stress parameters influenced by CuNPs compared to untreated SW480 cells(Control). A: total antioxidant capacity (TAC), B: total oxidative status (TOS), C: oxidative stress index (OSI). Data are expressed as mean ± SD(n=3). * $p < 0.05$ ** $p < 0.01$ and ***$p < 0.001$.

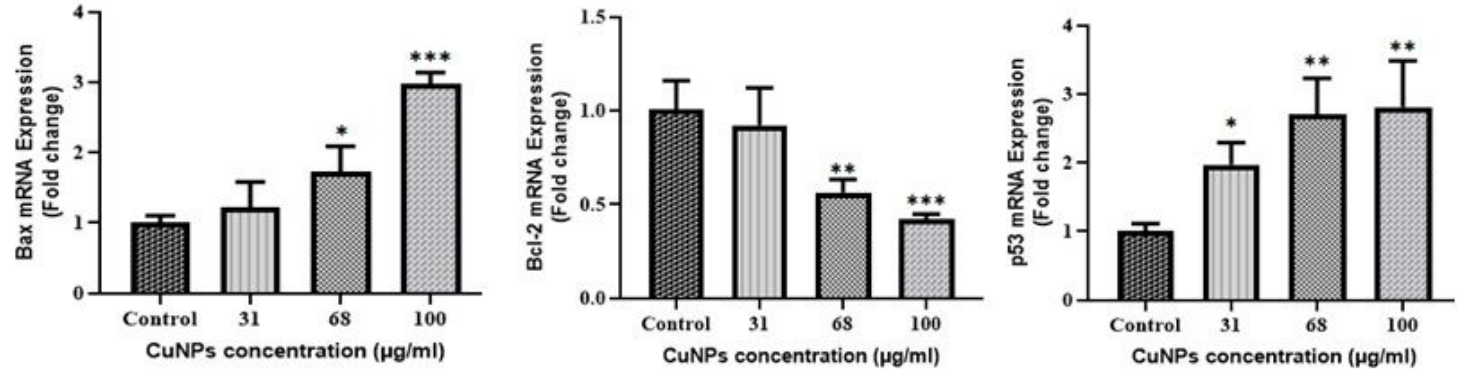

Figure 7

Bax, Bcl2, and P53 genes expression in SW480 cell line following CuNPs treatment for 24 h. Results are the mean ± SD (n=3) expressed as fold changes in mRNA expression. β-actin was used as the reference gene. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.