

Ferroptosis inhibitor alleviates cytotoxicity induced by cobalt nanoparticles through reactive oxygen species attenuation

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Research Article

Keywords: total hip arthroplasty, cobalt nanoparticles, ferroptosis, ferroptosis inhibitor, ROS

Posted Date: June 24th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-619496/v1>

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Abstract

Background Currently, tissue damage induced by cobalt nanoparticles (CoNPs) are the most noticeable adverse effect in the patients with metal-on-metal (MOM) hip prostheses. Therefore, effective therapeutic strategy is needed to limit the toxicity of CoNPs. In this study, we aimed to investigate the protective effect of ferroptosis inhibitor on CoNPs-induced cytotoxicity.

Methods To evaluate the detoxification effect of ferroptosis inhibitor, MG-63 cells were treated with CoNPs and ferroptosis inhibitor for 24 h. The cell viabilities were measured by CCK-8 assay. The expressions of glutathione peroxidase 4(GPX4) and solute carrier family 7 member 11(SLC7A11) were examined by western blot. Reactive oxygen species (ROS) and the GSH level were measured by the relevant kits. The cell apoptosis was detected by flow cytometry. The serum levels of inflammatory cytokines were measured with Elisa.

Results The cytotoxic effects of CoNPs were time- and dose-dependent. In addition, CoNPs elicited a significant ($p < 0.05$) reduction in cell viability and the glutathione (GSH) level with a concomitant increase in ROS generation and inflammatory cytokines such as tumour necrosis factor α (TNF- α), interleukin-1 β (IL-1 β) and interleukin-6 (IL-6) after 24 hours of exposure. Deferiprone (DFP) provided significant protection for cells after treatment with CoNPs. Western blot analysis demonstrated that CoNPs decreased the expression of GPX4 and SLC7A11, and treatment with DFP inhibited the decrease.

Conclusions CoNPs were highly cytotoxic and ferroptosis inhibitor DFP has a detoxification effect on cytotoxicity induced by CoNPs. Ferroptosis plays an important role in the process that CoNPs induced the cytotoxicity. The effectiveness of ferroptosis inhibition on MG-63 cells may initiate the development of novel strategies for reducing the adverse effects associated with metal implants in future.

Trial registration: no human participant.

Background

Total knee arthroplasty (TKA) and total hip arthroplasty (THA) are among the most effective orthopedic surgeries that can significantly restore joint function, relieve pain, and improve the patient's standard of living [1]. After total hip replacement, many different reasons lead to the production of metal particles, mainly due to mechanical wear and biological environmental erosion, such as erosion of the prosthesis by fluid and tissues around the hip joint. Cobalt-containing metal alloys release a large number of metal debris particles and metal ions[2]. Due to various of metal particles generated by wear, CoNPs may spread throughout the body, causing toxic effects around the implants. Horev-Azaria et al. believe that the toxicity of CoNPs is caused by the dissolution of Co^{2+} in the nanoparticles. However, previous researches have confirmed that the toxicity of nanoparticles themselves is greater than that of ionic forms [3, 4]. Many studies have shown that exposure to CoNPs can cause oxidative stress, lung inflammation and cell proliferation, which in turn leads to DNA damage and DNA mutations [5]. In vitro studies have indicated that CoNPs result in cellular oxidative stress [6] and genotoxicity [7]. Nanoparticles is known to enter

human cells and induce the production of reactive oxygen species, thereby leading to cytotoxicity and genotoxicity [8]. In the cytoplasm, CoNPs can affect the mitochondrial redox reaction, generate a large number of ROS, and consume intracellular antioxidants such as GSH, in turn, induce redox stress and cause damage to the cell structure [9]. Excessive ROS production leads to the damage of mitochondria, lysosomes and nuclei, and ultimately activates the apoptosis pathway [10]. In addition, pro-inflammatory cytokines such as TNF- α , IL-1 β , and IL-6 were found in the tissues surrounding the necrotic implants [11, 12]. Therefore, it is important to determine how to protect tissues from oxidative stress and inflammation.

The term ferroptosis was proposed by Dixon et al. in 2012 to describe the form of cell death induced by small molecules such as erastin [13]. Ferroptosis is dependent upon intracellular iron, accumulation of lipid ROS, and loss of activity of the lipid repair enzyme GPX4. Ferroptosis is a recently recognized form of regulated cell death, involving iron accumulation and lipid peroxidation, and its death phenotype is different from other types of cell death (such as apoptosis and necrosis) [14]. The depletion of GPX4, the down-regulation of SLC7A11 activity and the increase of lipid peroxidation products are common features of ferroptosis [15, 16]. At the same time, GPX4 is also an important regulator of ferroptosis [17]. The expression of SLC7A11 can potentially improve the survival rate of cells by regulating iron metabolism [18]. In addition, SLC7A11 is not only an essential component of the amino acid antiporter system Xc⁻, but also plays a crucial protective role in GSH synthesis. Since its recognition, ferroptosis has been involved in degenerative diseases such as the kidney and brain, and plays an important role in a variety of cancers [19]. The accumulation of reactive oxygen species plays a key role in causing ferroptosis [20], which is also the main trigger for the toxicity of CoNPs. In addition, ferroptosis inhibitors have been shown to have anti-inflammatory effects by inhibiting the expression of inflammatory cytokines including TNF- α [21], and the inflammatory response is also one of the toxic factors of CoNPs. Therefore, we speculate that ferroptosis inhibitors can reduce the toxicity of CoNPs based on inhibition of inflammation and ROS.

As far as we know, there is no previous study on ferroptosis inhibitors to antagonize the toxicity of cobalt nanoparticles. In this study, we explored the iron chelator DFP as an ferroptosis inhibitor to antagonize the toxicity of cobalt nanoparticles by depletion of ROS. This experiment aims to explore the possible protective effects of ferroptosis inhibitors on the toxicity of cobalt nanoparticles. Finally, we suggest that DFP, as an ferroptosis inhibitor, can be used as a therapeutic agent to prevent toxicity after MOM artificial joint replacement.

Materials And Methods

Reagent and chemicals

CoNPs (30nm,99.9%) were produced by Shanghai Chaowei Nano Technology Company. Dulbecco's Modified Eagle's medium (DMEM), 2', 7'-dichlorofluorescein diacetate (DCFH-DA), fetal bovine serum (FBS), trypsin-eDta and penicillin/streptomycin were purchased from Gibco (Life Technologies, Paisley, UK). Tissue culture dishes were obtained from Corning Inc. (New York, New York). Cell counting kit-8 (CCK-

8), ROS detection kit, were purchased from Beyotime Company (Jiangsu, China). Mouse TNF- α , IL-1 β , and IL-6 enzyme-linked immunosorbent assay (ELISA) kits were purchased from R&D Systems (Minneapolis, MN). Annexin V-FITC was purchased from Multi Sciences (Hanzhou, China). All antibodies were purchased from Abcam (Cambridge, UK). 10% polyacrylamide gel premix (NCM FastPAGE) was purchased from New Cell&Molecular Biotech Co.,Ltd (Suzhou, China). Deferiprone was gotten from MCE (New Jersey, USA).

Preparation of Cobalt nanoparticles

CoNPs samples were sterilized at 180°C for 4 h, suspended in ultrapure water to achieve the initial concentration of 40 mM. The Freshly prepared stock solution was ultrasonicated at room temperature at 40 W for 30 min in order to minimize particle aggregates and diluted to achieve the required concentrations (25 μ M, 50 μ M, 100 μ M, 200 μ M, 400 μ M, 800 μ M) with complete culture medium.

Preparation of DFP

DFP (1mM) was irradiated and sterilized under ultraviolet light for 2h, and then diluted to 25 μ M with basal medium for subsequent experiments. Store the processed DFP at -20°C.

Cell culture and sample treatment

The MG-63 cell line was bought from the cell repository of Chinese Academy of Sciences. Briefly, cells were cultured in DMEM supplemented with 10% FBS, penicillin and streptomycin. The medium was replaced every 3 days. All cells were grown at 37°C in a humidified incubator containing 5% CO₂. For the dose-related experiments, the cells were exposed to CoNPs for 4, 24 and 48 h. To examine the protective effects of DFP on the viability of cells, the cells were treated with varying concentrations of DFP and exposed to target concentration of CoNPs for 24 h.

Cell Viability Assay

According to the manufacturer's protocol, MG-63 cells were incubated in 96-well plates and then treated with different concentrations of DFP and 400 μ M CoNPs for 4, 24 or 48 h. After that, 10 μ L CCK-8 was added to each well and incubated at 37 °C for another 4 h. The absorbance was measured by the microplate reader at 450 nm. The Cell viability ratio was expressed as percent control. From this, the 50 % inhibitory concentration (IC₅₀ value)[22] was calculated.

Detection of Cytokines

Cells were plated at a density of 1 \times 10⁵ cells/well in six-well plates and incubated for 24 h at 37°C. Then the cells were exposed to CoNPs (400 μ M) with or without the treatment of DFP (25 μ M) for 24 h. After treatment, cytokines levels of the supernatants collected from cell cultures were measured. The concentrations of TNF- α , IL-1 β , IL-6 in the culture media were determined using ELISA kits according to the manufacturer's instructions. Each sample was analyzed in triplicate.

ROS measurement

Spectrofluorometry analysis and fluorescence microscopy imaging were used to assess ROS generation in cells exposed to target concentrations of CoNPs and the protective effects of DFP treatment. For the

spectrofluorometry analysis, cells (1×10^5 /well) were seeded in 96-well plates. MG-63 cells were exposed to CoNPs and ferroptosis inhibitor for 24 h. Following completion of the DCFH-DA reaction, the reaction mixture was washed thrice by 200 ml of PBS in each well. The fluorescence intensity of the suspension was measured on a multi-well microplate reader using excitation and emission of 485 nm and 525 nm, respectively. Values were expressed as the percentage of fluorescence intensity relative to the control wells.

GSH Measurement

The cells were treated with 25 μ M DFP and exposed to 400 μ M CoNPs for 24 h. After the exposure, the cells were washed with PBS twice, scraped off, suspended in PBS, and centrifuged at 1000 \times g. The cell pellet was then homogenized in 5% 5-sulfosalicylic acid. Next, the suspension was lysed by freezing and thawing twice after 5 min centrifuged at 10,000 \times g for 10 min. The amount of GSH were measured according to the manufacturer's protocol.

Quantitative assessment of cell apoptosis detected by flow cytometry

For measurement of cell apoptosis, the AnnexinV-FITC assay kit was conducted according to manufacturer's protocol. Briefly, cells were washed with PBS solution and harvested. Next, the cells were suspended in binding buffer at 1×10^6 cells/ml. A 100 ml cell suspension was withdrawn supplemented with 5 ml AnnexinV-FITC and 10 ml of PI and subsequently incubated for 15 min at room temperature in the dark. A Becton Dickinson flow cytometer was employed for the measurement within 1 h of staining.

Western Blot

The cells were treated with 25 μ M DFP and exposed to target concentrations of CoNPs for 24 h. Cells were washed in cold PBS and lysed in RIPA with 1 mM phenylsulfonyl fluoride (PMSF, beyotime, Nantong, China). The supernatants were harvested and protein concentrations were measured with the bicinchoninic acid (BCA) assay kit. The concentrated samples were subjected to 15% SDS-polyacrylamide gel electrophoresis and transferred to a poly-vinylidene difluoride filter (PVDF) membrane by a transfer apparatus at 300 mA for 1 h. The membranes were blocked for 1 h and then incubated with primary antibodies at 4 $^{\circ}$ C overnight. After washing with TBST, the membranes were incubated with secondary antibodies for 2 h at room temperature. The PVDF membranes were washed four times with TBST according to the manufacturer's protocol.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism software 7 (GraphPad Software Inc., La Jolla, CA, USA) using one-way analysis of variance (ANOVA) followed by Dunnett's test to evaluate significance relative to control. Results shown summarise data from three independent experiments and were represented as the mean \pm standard deviation (SD). Differences between groups were considered significant if p-values < 0.05.

Results

Ferroptosis inhibitor protected against cytotoxicity induced by CoNPs in MG-63 cells

Cells were treated with different concentrations of CoNPs for 4h, 24h or 48h and the effect of CoNPs on MG-63 cell survival was determined by CCK-8 assay. The percentage of survived cells strikingly decreased with the increase of CoNPs concentration (Fig. 1a). The IC₅₀ value was 400 μ M 24 h later, respectively. Cells cultured in the medium with varying concentrations of DFP, which showed a dose-dependent survival rate. When the concentration of DFP increased, the viability of MG-63 cells increased. When cells were cultured in 400 μ M CoNPs and different concentrations of DFP, the viability of cells was significantly higher compared with the cells treated with CoNPs (Fig. 1b).

Ferroptosis inhibitor reduced inflammatory cytokines in MG-63 cells

The results are shown in the Fig. 2. The levels of TNF- α , IL-1 β and IL-6 in the CoNPs group were noticeably higher than those in the control group. Compared with the CoNPs group, DFP treatment had a significant protective effect, and significantly reduced TNF- α ($p < 0.05$), IL-1 β ($p < 0.05$) and IL-6 ($p < 0.05$) induced by cobalt nanoparticles expression.

ROS formation in MG-63 cells

The oxidative damage induced by ROS is considered to be an important cause of the toxicity of cobalt nanoparticles. ROS is also the main incentive of ferroptosis. In this study, as shown in the Fig. 3, after 400 μ M CoNPs were treated for 24 h, the ROS level of MG-63 cells increased significantly ($p < 0.05$), while DFP (25 μ M) could significantly inhibit this increase ($p < 0.05$), and the difference was statistically significant ($p < 0.05$).

GSH is the key cellular endogenous antioxidant that can scavenge toxic free radicals, and depletion of GSH appears to promote intracellular ROS accumulation, leading to apoptosis. Altered GSH levels represent increased cellular response to oxidative stress. The results suggested that CoNPs treatment decreased GSH levels ($p < 0.05$) and DFP inhibited this decrease ($p < 0.05$) as measured by the GSH assay kit (Fig. 3c).

Apoptosis assay in MG-63 cells

To investigate the apoptosis rate of cells treated with 400 μ M CoNPs and 25 μ M DFP, the AnnexinV-FITC assay kit was used. Incubation with 400 μ M CoNPs for 24 h mildly increased the cell apoptosis rate compared with the control group. When DFP was added as a treatment before exposure to CoNPs, the apoptosis was slightly alleviated (Fig. 4a, b).

Ferroptosis inhibitor exerted the positive regulation on GPX4 in MG-63 cells

GPX4 is the central regulator of ferroptosis, and the decline of GPX4 is often used as a marker of ferroptosis[23–25]. SLC7A11 is a unit of the glutamate-cystine antiporter Xc⁻, resulting in enhanced lipid oxidation and ferroptosis. Western blot showed that CoNPs noticeably decreased the protein amount of GPX4 and SLC7A11. However, the decrease in GPX4 and SLC7A11 levels was inhibited when cells were treated with 25 μ M DFP and 400 μ M CoNPs for 24 h. These results suggest that CoNPs might induce ferroptosis and the ferroptosis inhibitor DFP exerts therapeutic action against CoNPs.

Discussion

With the gradual popularity of artificial joint replacement, the materials of artificial joint prostheses have also attracted much attention. Due to the low dislocation and wear rate of MOM implants, their use in prostheses has gradually become widespread. It is crucial to elucidate the adverse biological consequences of metal nanoparticles released from implants, and provide a potential method for reducing the adverse effects associated with these metal particles. The main adverse effects come from cobalt nanoparticles produced under long-term wear and tear. So far, the mechanism of CoNPs-induced toxicity has not been fully understood, and the current mainstream is still the ROS theory and the inflammation caused by ROS[26, 27]. The excessive accumulation of ROS and the production of a large number of inflammatory cytokines such as TNF- α play an important role in the toxicity mechanism induced by cobalt nanoparticles, leading to inflammation, cell damage and proliferation, which in turn leads to DNA damage and DNA mutations, as well as damage to mitochondria, lysosomes and the nucleus, eventually leading to cell death. Past studies have found that although the apoptosis rate of treatment is significantly higher than that of the control group, it is still much lower than the cell death rate[28]. Therefore, it could be possible that there are still other types of cell death besides apoptosis in MG-63 cells exposed to CoNPs. Ferroptosis is a newly identified form of cell death that is characterized by the accumulation of intracellular iron and the need for lipid peroxidation[14]. In this process, a variety of inducers disrupt the cell redox balance and produce a large amount of lipid peroxidation products, which ultimately trigger cell death. Generally speaking, ferroptosis is a form of ROS-dependent regulatory necrosis that occurs through multiple signals and pathways[29]. In this study, we focused on the role of ferroptosis in toxicity induced by cobalt nanoparticles and evaluate the protective effects of ferroptosis inhibitors on MG-63 cells.

In current study, the CCK8 was used to assess the viability of MG-63 cells exposed to CoNPs. The results showed that the cytotoxic effect of CoNPs on MG-63 cells was time- and dose-dependent. On this basis, the IC50 value was calculated, and 400 μ M CoNPs was used as a positive control for subsequent experiments. In addition, 25 μ M DFP was selected to evaluate its effect on CoNPs-induced cell damage. Our results suggest that DFP treatment can reduce the decline in cell viability induced by CoNPs. The cell viability of the experimental group treated with the ferroptosis inhibitor was much higher than that of the control group, which indicated that the ferroptosis inhibitor had a certain protective effect on MG-63 cells.

Studies have shown that cobalt-chromium wear particles can induce the release of a variety of pro-inflammatory cytokines, such as TNF- α , IL-1 β , and IL-6[30–32]. In this study, we confirmed the pro-inflammatory effect of CoNPs through the increase of TNF- α , IL-1 β and IL-6, and determined whether ferroptosis inhibitors can reduce the increase in the levels of inflammatory cytokines induced by CoNPs. The treatment of ferroptosis inhibitor significantly reduced the inflammatory cytokines induced by CoNPs after 24 hours of exposure, and the difference was statistically significant compared with the control group ($P < 0.05$). DFP treatment can significantly reduce the expression of TNF- α , IL-1 β and IL-6 in MG-63 cells induced by 400 μ M CoNPs, suggesting that ferroptosis inhibitors have a protective effect on inflammatory cytokines in MG-63 cells induced by 400 μ M CoNPs.

CoNPs induced a noticeable increase in ROS. ROS is considered to be the main cause of ferroptosis, which can cause DNA damage, oxidative stress and activate other cellular processes. On the one hand, ROS acts as a dynamic balance signal molecule that regulates cell growth, and on the other hand, it acts as an adaptive response at physiological concentrations. However, the production of ROS at higher concentrations can cause cell damage and death. GSH is an important antioxidant substance that plays an important role in protecting against apoptosis. The depletion of GSH promotes the accumulation of ROS in cells and cell apoptosis. In current study, compared with CoNPs group, the addition of ferroptosis inhibitors significantly reduced the production of ROS in MG-63 cells (Fig. 3a, b). In addition, our results shows that CoNPs treatment decreased GSH levels and DFP reversed this decrease ($p < 0.05$) as measured by the GSH assay kit (Figure.3c).

Flow cytometry results exhibited that CoNPs induced mild cellular apoptosis, which could be further alleviated by the exposure of DFP. Although the apoptosis rate of MG-63 cells for the CoNPs treatment group was significantly increased as compared to the control group (Fig. 4), it was still much lower than that of control group, as shown by CCK-8 under the same conditions (Fig. 1). These results indicate that non-apoptotic mechanisms are involved in CoNPs-mediated cell death.

GPX4 and SLC7A11 are recognized as important endogenous regulators of ferroptosis and the decline of GPX4 and SLC7A11 is always regarded as a marker of ferroptosis[33, 34]. A previous study has indicated that SLC7A11 protects cells from ferroptosis by increasing GSH production. In this study, we found that after exposure to CoNPs, the protein levels of GPX4 and SLC7A11 were significantly decreased, while DFP up-regulated the levels of GPX4 and SLC7A11, reducing the damage of ROS. Our results strongly support the idea that ferroptosis inhibitors can upregulate the expression of GPX4 and SLC7A11 to inhibit the cytotoxicity caused by CoNPs.

Conclusions

In summary, all these results strongly supported the idea that the ferroptosis inhibitor counteracts cytotoxicity and inflammation induced by CoNPs and ferroptosis plays an important role in the occurrence of CoNPs-induced toxicity. In addition, we are supposed to compare the results of different ferroptosis inhibitors on cell detoxification, and further screen out the most suitable drugs to relieve the CoNPs-induced toxicity produced in MOM artificial joints. The research provides a new perspective for antagonizing CoNPs-induced cytotoxicity, and provides a potential method to reduce adverse reactions related to MOM joint implants.

Abbreviations

CoNPs: cobalt nanoparticles; MOM: metal-on-metal; GPX4: glutathione peroxidase 4; SLC7A11: solute carrier family 7 member 11; ROS: Reactive oxygen species; GSH: glutathione; TNF- α : tumour necrosis factor α ; IL-1 β : interleukin-1 β ; IL-6: interleukin-6; DFP: Deferiprone; TKA: Total knee arthroplasty; THA: total hip arthroplasty; DCFH-DA: 2', 7'-dichlorofluorescein diacetate; DMEM: Dulbecco's Modified Eagle's

medium;FBS: fetal bovine serum; CCK-8: Cell counting kit-8; PMSF: phenylsulfonyl fluoride; PVDF: polyvinylidene difluoride filter;SD: standard deviation

Declarations

Competing interests: The authors declare that they have no conflicting interests.

Funding: This work was supported by Postgraduate Research & Practice Innovation Program of Jiangsu Province [grant number SJCX20_1167].

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Authors' contributions: CW and WZ researched literature and conceived the study. YL was involved in protocol development and data analysis. CW wrote the first draft of the manuscript. All authors reviewed and edited the manuscript and approved the final version of the manuscript

Acknowledgements: Not applicable

Availability of data and materials: The datasets used and/or analyzed during the current study are not publicly available. Data are however available from the corresponding author on reasonable request.

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Figures

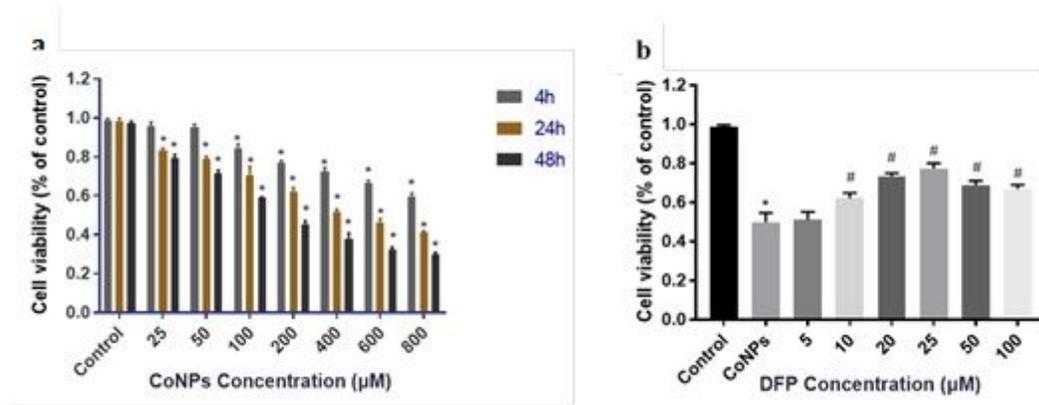


Figure 1

Changes in cell viability of MG-63 cells following CoNPs and DFP treatment. Cell viability of MG-63 cells exposed to 400 µM CoNPs and different concentrations of DFP for 24 h, as determined by a Cell Counting kit 8 assay. Data are presented as the mean ± standard deviation of three independent experiments performed in triplicate. * $p < 0.05$ vs. control, # $p < 0.05$ vs. CoNPs group.

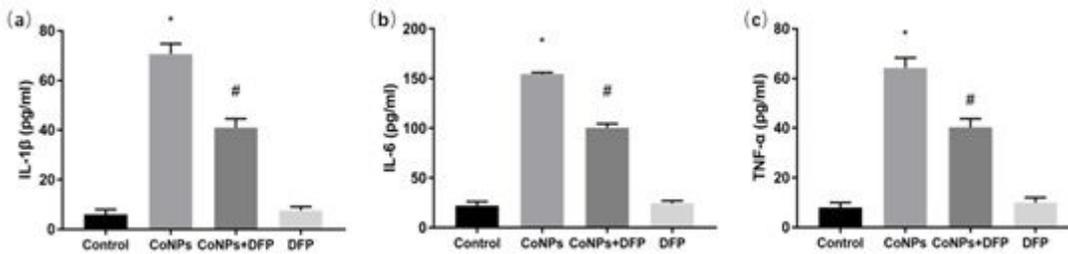


Figure 2

Effects of DFP on pro-inflammatory cytokines and anti-inflammatory cytokines expression in cells in response to exposure to CoNPs. IL-1β (a), IL-6 (b), TNF-α (c) levels in the supernatants of tissue cultured cells after 24 h of incubation, as evaluated by ELISA. The results are shown as the mean ± SD of three independent experiments performed in triplicate. * $p < 0.05$ compared to control group; # $p < 0.05$ compared to CoNPs group.

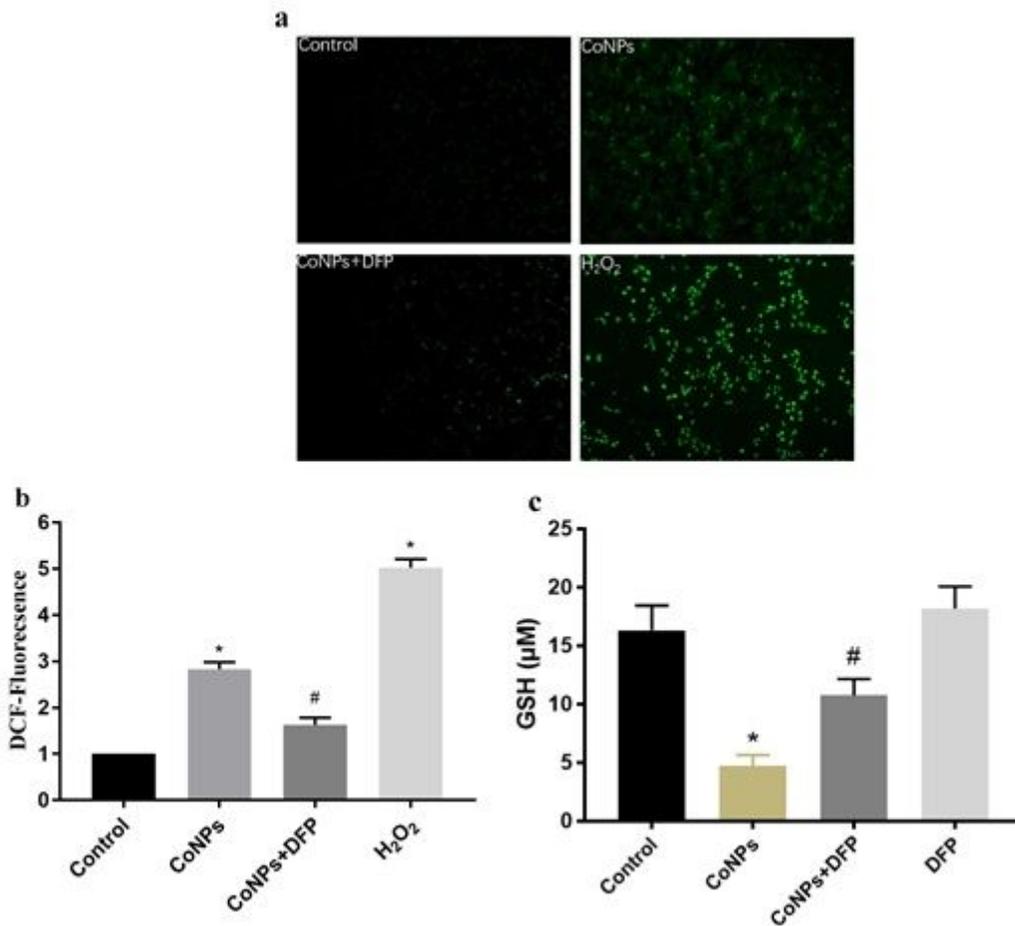


Figure 3

CoNPs treatment enhanced the level of intracellular ROS in MG-63 cells, while DFP significantly inhibited the formation of ROS. The cells were exposed to CoNPs (400 µM) and DFP (25 µM) for 24 h. a Representative microphotographs (magnification, x40) demonstrating CoNP-induced ROS generation in MG-63 cells treated with control, 400 µM CoNPs, 400 µM CoNPs + 25 µM DFP and H₂O₂ (24 h). H₂O₂ group as positive control. b The fluorescent intensity of cells with different treatments. c Effects of CoNPs and DFP on GSH in MG-63 cells. **p* < 0.05 vs. control, #*p* < 0.05 vs. CoNPs group.

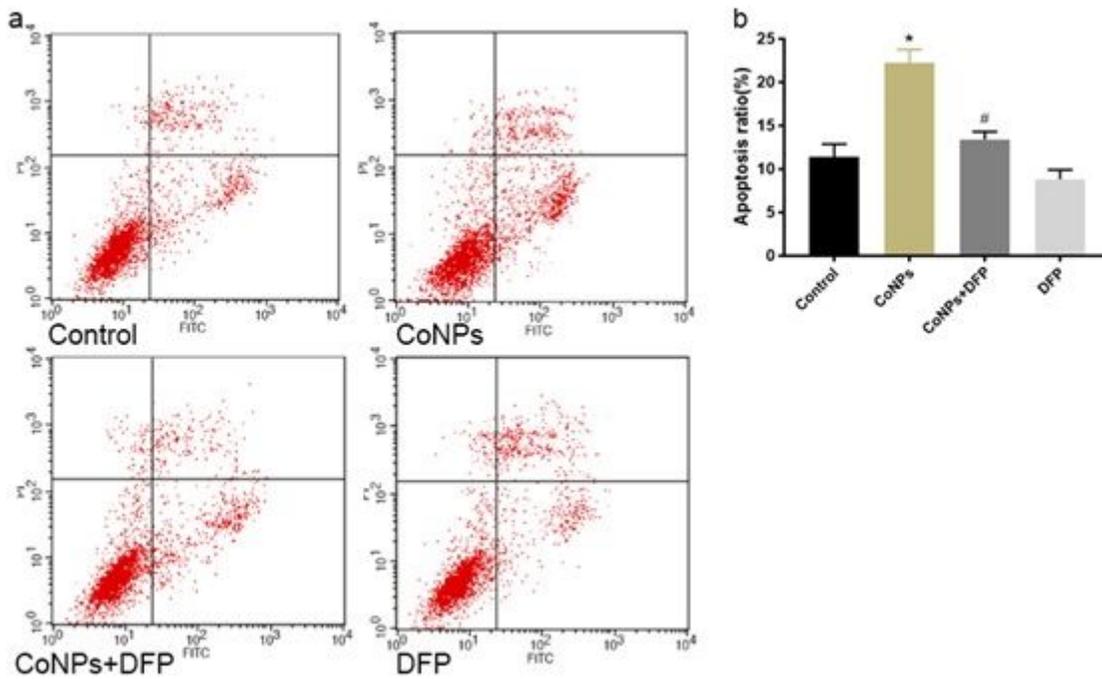


Figure 4

Cell apoptosis levels of MG-63 cells in presence of CoNPs and DFP. The cells were co-incubated with CoNPs (400 μ M) and DFP (25 μ M) for 24 h. a The apoptosis was detected by flow cytometry. b Quantification of MG-63 cells apoptosis. All the data were expressed as mean \pm SD of three independent experiments performed in triplicates. * $p < 0.05$ vs. control, # $p < 0.05$ vs. CoNPs group.

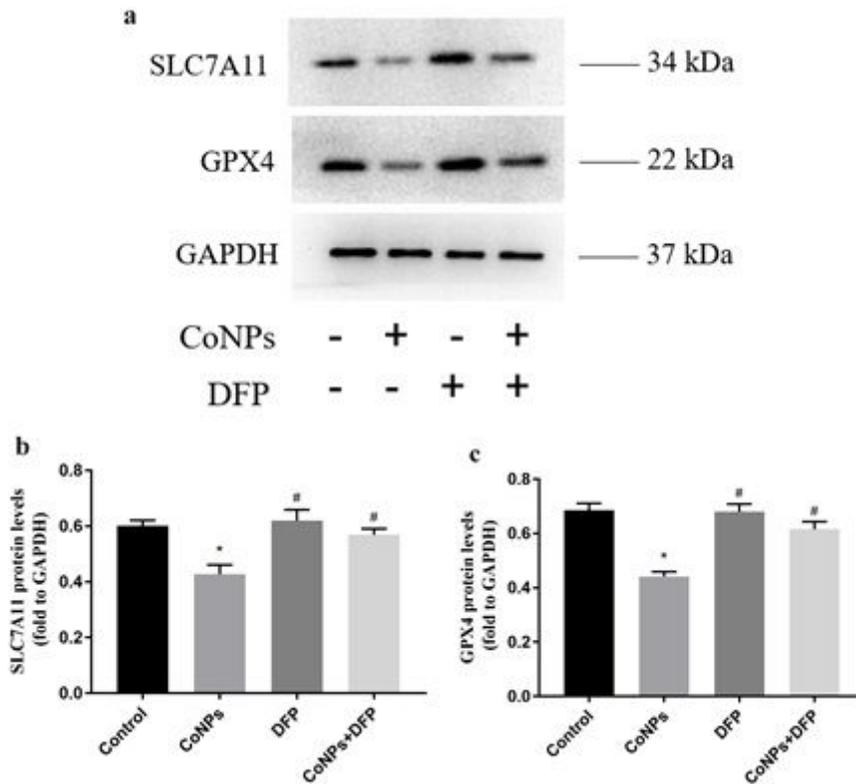


Figure 5

Effects of DFP on the protein levels of GPX4 and SLC7A11 in MG-63 cells after exposed to CoNPs. a Expressions of GPX4 and SLC7A11 were evaluated by western blotting. b Quantification of GPX4 and SLC7A11 expressions. * $p < 0.05$ vs. control, # $p < 0.05$ vs. CoNPs group.