

Melatonin counteracts cobalt nanoparticle-induced cytotoxicity and genotoxicity by deactivating reactive oxygen species-dependent mechanisms in the NRK cell line

YAKE LIU*, XIAOYOU YANG*, WEI WANG, XUEFEI WU, HAI ZHU and FAN LIU

Department of Orthopedics, The Affiliated Hospital of Nantong University, Nantong, Jiangsu 226001, P.R. China

Received October 24, 2016; Accepted June 9, 2017

DOI: 10.3892/mmr.2017.7309

Abstract. Cobalt nanoparticles (CoNPs) released from metal-on-metal implants have caused considerable concern. Oxidative stress is associated with the mechanism underlying cobalt-induced cytotoxicity and genotoxicity. The indolamine melatonin exhibits protective effects against damage induced by metals. The present study investigated the *in vitro* effects of melatonin on the cytotoxicity and genotoxicity induced by CoNPs. CoNPs (20-50 nm in diameter) were employed in the present study. NRK rat kidney cells were exposed to various concentrations of CoNPs for different durations. The results of the current study demonstrated that CoNPs significantly increased reactive oxygen species (ROS) production and reduced cell viability, as determined by dichlorofluorescein diacetate, and Cell Counting Kit-8 and lactate dehydrogenase leakage assays, respectively. Furthermore, western blot analysis demonstrated that CoNPs led to an increase in the ratio of Bcl-2-associated X/Bcl-2, and the expression of cleaved caspase-3 was upregulated, which indicated increased apoptosis levels. Genotoxicity was detected by a comet assay, which revealed a significant induction in DNA damage, as determined by increases in the tail DNA % and olive tail moment. Phosphorylated-histone H2AX foci analyses by immunofluorescence also demonstrated that CoNPs induced DNA-double strand breaks. However, cellular treatment with melatonin reduced the effects of CoNPs on NRK cells by reducing the production of ROS. The results of the present study demonstrated that CoNPs induced cytotoxicity and genotoxicity by increasing oxidative stress, and melatonin may have pharmacological potential in protecting against the damaging effects of CoNPs following total hip arthroplasty.

Introduction

Metal-on-metal (MoM) resurfacing hip implants have received recognition since the second generation was developed in 1990. Cobalt-chromium (CoCr) alloy casting implants have exhibited numerous benefits; they reduce the risk of dislocation and maintain improved femoral loading, and reduce stress-shielding and exhibit improved wear characteristics, which contributes to joint stability and the avoidance of component impingement (1). However, potential complications due to the dissemination of metal particles in the nanometer size range, and concomitant release of ions throughout the circulation, have limited the success of such implants (2).

During revision surgeries or necropsy examinations, allochroic tissue is commonly observed around CoCr implants. Furthermore, certain patients experience inexplicable pain, which may be associated with the tissue damage caused by MoM hip replacements wear (3). Increasing evidence indicates that these particles, and concomitant ions, may be associated with various adverse health issues, including neurological (4) (static tremor, motor incoordination, vertigo, hearing decline and visual issues), cardiac (5) (myocardiopathy) and endocrine (6) symptoms.

A high number of these nanometer-sized particles are present, and they are phagocytosed by macrophages, which leads to the release of various types of mediators, the majority of which are free radicals (7). This reactive oxygen species (ROS) generation induced by metal nanoparticles was reported to be closely associated with apoptosis (8), which may occur via the oxidation of cellular structures and components. Ladon *et al* (9) reported an increase in chromosome aberrations consisting of chromosome translocations in the peripheral blood following hip arthroplasty. Similar genotoxic potential was also observed when exposed to CoCr nanoparticles (10). Furthermore, accumulating evidence indicates that ROS has an important role in the cytotoxicity process as well as in genotoxicity (11-13).

Melatonin, the primary product that is secreted by the pineal gland, is a ubiquitous biological signaling and functionally diverse molecule. The indolamine has been reported to be beneficial in various oxidative stress-associated diseases, including diabetes (14), retinopathy (15), cardiovascular (16) and genital (17) diseases, and cancer (18), with no toxicity cases reported. Compared with conventional antioxidants,

Correspondence to: Professor Fan Liu, Department of Orthopedics, The Affiliated Hospital of Nantong University, 19 QiXiu Road, Nantong, Jiangsu 226001, P.R. China
E-mail: liufan20142016@aliyun.com

*Contributed equally

Key words: cobalt nanoparticles, cytotoxicity, genotoxicity, oxidative stress, melatonin

such as vitamin C and vitamin E, melatonin exhibits an increased ability to scavenge free radicals (19,20). Limited data regarding the effects of melatonin on CoNP-induced is available. Therefore, the present study investigated whether melatonin exhibits protective effects on CoNP-induced cytotoxicity and genotoxicity in the NRK cell line.

Materials and methods

Cell culture. NRK cell line (purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China) derived from rat kidney were routinely cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) supplemented with 10% (v/v) fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 1% penicillin-streptomycin (penicillin, 100 U/ml; streptomycin, 100 µg/ml; Sigma-Aldrich; Merck KGaA) at 37°C under a humidified atmosphere of 5% CO₂ in a cell culture incubator. Cells (1.5x10⁴ cells/well) were exposed to varying concentrations of CoNPs (diameter, 20-50 nm; Sigma-Aldrich; Merck KGaA; 10, 50, 100, 200, 400 and 500 µM) in media for different durations (4, 24 and 48 h) for subsequent experiments at 37°C. Cells cultured with DMEM acted as the control group. An appropriate concentration of CoNPs (50% inhibitory concentration, IC₅₀; 100 µM) was selected for experiments that involved melatonin treatment. Melatonin (Sigma-Aldrich; Merck KGaA) was dissolved in dimethylsulfoxide (DMSO; Sigma-Aldrich; Merck KGaA) prior to use and was immediately added to cells at various doses (5, 10, 50, 100, 200 and 300 µM), which was maintained during treatment with CoNPs, and was also performed for various durations (4, 24 and 48 h) at 37°C. DMSO was used as a vehicle and the final concentration did not exceed 0.1% (v/v) (21).

Nanoparticles. Stock solutions of CoNPs were prepared at a concentration of 100 mM in ultrapure water. The stock solutions were sonicated intermittently six times for 30 min, as described previously (22). The stock solutions were freshly diluted with culture medium to the test concentrations (10, 50, 100, 200, 400 and 500 µM).

Cell viability assay. Cell viability was detected using the Cell Counting kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Cells (1.5x10⁴ cells/well) were plated into 96-well plates and exposed to CoNPs (0, 10, 50, 100, 200, 400 or 500 µM) and melatonin (0, 5, 10, 50, 100, 200 or 300 µM) for 4, 24 or 48 h. Culture medium served as the control in each experiment. In order to investigate the effects of melatonin on the viability of NRK cells exposed to CoNPs, the cells were exposed to 100 µM (IC₅₀) CoNPs, and subsequently, the cells were incubated for 4, 24 or 48 h in the presence of melatonin (0-300 µM). CCK-8 solution (10 µl) was added to each well of the plate for 30 min at 37°C prior to detection. The CCK-8 formazan product was measured at 450 nm using a microplate reader.

Lactate dehydrogenase (LDH) leakage assay. The release of cytoplasmic LDH enzyme into the culture medium was determined, as previously described (13). NRK cells exposed

to 100 µM CoNPs were treated with melatonin (10 or 100 µM) for 4, 24 and 48 h. Following exposure, 120 µl supernatant from the centrifuged culture media were collected (200 x g for 3 min at room temperature). The LDH activity was assayed in 3 ml reaction mixture with 100 µl pyruvic acid (2.5 mg/ml phosphate buffer) and 100 µl reduced nicotinamide adenine dinucleotide (NADH; 2.5 mg/ml phosphate buffer). The remaining volume was adjusted with phosphate buffer (0.1 M; pH 7.4). The rate of NADH oxidation was determined at a wavelength of 490 nm using a microplate reader. The amount of LDH released was expressed as LDH activity (IU/l) in culture media.

Western blot assay. Following CoNP (100 µM) exposure, cells treated with control and melatonin (10 or 100 µM) for 24 h were harvested, pelleted (200 x g for 3 min at room temperature) and lysed by adding ice-cold radioimmunoprecipitation assay lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 0.1% Triton-X-100, 10% sodium deoxycholate, 10% SDS, 1 mM NaF and protease inhibitor cocktail. Protein concentrations were determined by the Bradford method. Total protein (30 µg) was resolved by 10 and 12% SDS-PAGE and transferred onto polyvinylidene fluoride membranes. The blotted membrane was blocked with 5% non-fat dry milk for 2 h at room temperature and incubated overnight at 4°C with specific antibodies against Bcl-2-associated X (Bax; 1:800; cat. no. D3R2M), Bcl-2 (1:800; cat. no. D17C4), cleaved caspase-3 (1:500; cat. no. 9654) and GAPDH (1:1,000; cat. no. 8884). All primary antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). The blots were subsequently incubated with horseradish peroxidase-conjugated secondary antibody (1:1,000; cat. no. A0545; Sigma-Aldrich; Merck KGaA) for 2 h at room temperature and bands were detected by enhanced chemiluminescence using SuperSignal West Femto Chemiluminescent Substrate (Pierce; Thermo Fisher Scientific, Inc.). Densitometric analysis was performed by ImageJ software (version 1.47; National Institutes of Health, Bethesda, MD, USA).

Comet assay. DNA damage detected by comet assay was an adaptation described by Uzar *et al.* (23). Cells (2x10⁴ cells/well) exposed to CoNPs were co-incubated with melatonin (10 or 100 µM) for 4, 24 or 48 h, and washed with PBS, trypsinized, centrifuged at 200 x g for 3 min at room temperature and resuspended in 0.5 ml PBS. Briefly, 100 µl cells were mixed with 100 µl pre-warmed low-melting point agarose (0.65% in PBS), layered on normal-melting point agarose (1.5% in distilled water) precoated microscope slides and covered with a coverslip. Slides were placed in a refrigerator for solidification, coverslips were removed and slides were incubated for 1 h at 4°C in lysis solution (2.5 M NaCl, 100 mM EDTA and 10 mM Tris-HCl, pH 10) with 10% DMSO and 1% Triton-X-100. DNA was denatured for 20 min in cold-fresh electrophoresis buffer (0.3 M NaOH, 1 mM EDTA, pH 13) at 4°C and electrophoresis was performed at 4°C for 20 min (20 V/300 mA). Following electrophoresis, slides were neutralized with 0.4 M Tris-HCl buffer (pH 7.5) three times for 5 min. Cells were scored under a fluorescent microscope at x400 magnification by using ImageJ software (version 1.47) immediately after DNA was stained with ethidium bromide (20 µg/ml) for 15 min at room

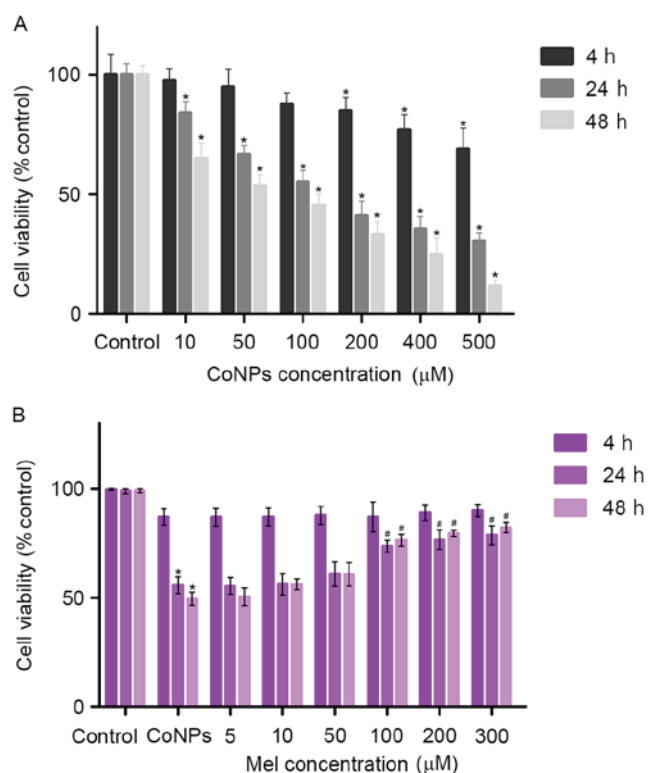


Figure 1. Changes in cell viability of NRK cells following CoNP and melatonin treatment. (A) Viability of NRK cells exposed to 0-500 μM CoNPs for 4, 24 and 48 h, as determined by a Cell Counting kit-8 assay. (B) Effects of 0-300 μM melatonin on cell viability in NRK cells when exposed to 100 μM CoNPs for 4, 24 and 48 h (B). Data are presented as the mean \pm standard deviation of three independent experiments performed in triplicate. * $P < 0.05$ vs. control and # $P < 0.05$ vs. CoNPs group at same time point. CoNPs, cobalt nanoparticles; mel, melatonin.

temperature. At least 100 cells were scored per concentration. The percentage tail DNA, tail length and olive tail moment (OTM; product of tail length and the fraction of total DNA in the tail) was analyzed.

Immunostaining of phosphorylated-histone 2AX (γH2AX) foci. γH2AX foci were used as a marker of DNA-double strand breaks (DSBs). NRK cells (1×10^5 cells/well) were cocultured with 100 μM CoNPs and melatonin (10 or 100 μM) for 4, 24 and 48 h. Cells were subsequently reseeded on coverslips in 6-well plates, fixed for 15 min with 4% paraformaldehyde at 4°C and washed with PBS. Following treatment with 0.2% Triton-X-100 for 15 min at 4°C, blocking was performed by incubation for 90 min at room temperature with blocking solution containing 3% (v/v) bovine serum albumin (Sigma-Aldrich; Merck KGaA) in PBS. Rabbit polyclonal antibody (1:500 in blocking solution; cat. no. ADI-905-771-100; Alexis Biochemicals; Enzo Life Sciences, Inc., Farmingdale, NY, USA) against human γH2AX was incubated with cells overnight at 4°C. The following day, the coverslips were washed three times with PBS and secondary donkey anti-rabbit Cy3-labeled antibody (1:500 in blocking solution; cat. no. AP182C; Chemicon; EMD Millipore, Billerica, MA, USA) was incubated with cells for 60 min at 37°C. The coverslips were finally inverted onto pre-cleaned dry slides with a droplet (30 μl per coverslip)

of DAPI-Vectashield® Antifade Mounting Medium (Vector Laboratories, Inc., Burlingame, CA, USA) and edges were sealed with clear nail polish. All slides were examined using an inverted fluorescence microscope (magnification, x40). The protocol described in the present study is an adaptation of the protocol described by Tsousi *et al* (24). For quantitative analysis, foci were counted using ImageJ software (version 1.47). For each sample at least 60 cells were analyzed.

Determination of intracellular ROS content. Intracellular ROS levels were detected based on the ROS-mediated oxidation of nonfluorescent dichlorofluorescein (DCFH), which leads to the production of fluorescent dichlorofluorescein (DCF). DCFH-diacetate (DCFH-DA) passively diffuses into cells and esterases deacetylate DCFH-DA, leading to the formation of nonfluorescent DCFH. When ROS is present, DCFH undergoes a reaction with ROS that leads to the production of fluorescent DCF, which cannot exit cells. Therefore, the level of fluorescence is associated with ROS levels within cells (25). After cells (2×10^4 cells/well) were exposed to 100 μM CoNPs in 96-well plates, various concentrations (10 or 100 μM) of melatonin were added for 4, 24 and 48 h. Cells were subsequently incubated with DCFH-DA (10 μM ; Nanjing Jiancheng Bioengineering Institute, Nanjing, China) at 37°C for 20 min. The fluorescence of the cells in each well was measured at an emission wavelength of 530 nm and an excitation wavelength of 485 nm using microplate reader. ROS levels were expressed as a percentage of the control.

Statistical analysis. Data are presented as the mean \pm standard deviation. Significant differences were evaluated by one-way analysis of variance followed by Dunnett's post hoc test. Statistical analyses were performed with SAS 9.1 statistical software (SAS Institute, Cary, NC, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

CCK-8 and LDH leakage assays. Cell viability was assessed after 4, 24 and 48 h exposure to 0, 10, 50, 100, 200, 400 and 500 CoNPs, and significant decreases in cell viability were observed in the 24 and 48 h treatment groups, even at low concentrations of CoNPs, compared with the control group at the same time point ($P < 0.05$; Fig. 1A). At concentrations of 200, 400 and 500 μM , the cell viability in the 4 h exposure group was significantly reduced compared with the 4 h control group ($P < 0.05$; Fig. 1A).

No significant protective effects were observed when cells were treated with 5, 10 or 50 μM melatonin (Fig. 1B). However, significant increases in cell viability were observed in the 24 and 48 h treatment groups when treated with 100, 200 and 300 μM melatonin ($P < 0.05$; Fig. 1B), compared with the CoNP-only treatment group (100 μM). To verify the effective concentration of melatonin, 10 μM was selected as the low concentration and 100 μM as the high concentration for subsequent experiments.

The protective effects of melatonin against reductions in cell viability induced by CoNPs observed in the CCK-8 assay were also supported by similar results obtained from the LDH leakage assay (Fig. 2).

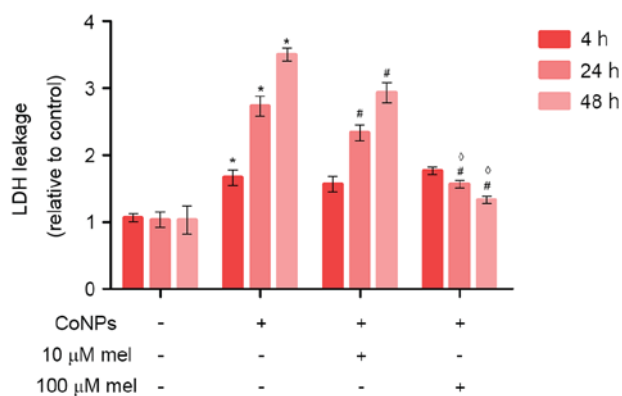


Figure 2. Analysis of cytotoxicity detected by LDH leakage in NRK cells following exposure to 100 μM CoNPs and melatonin (10 and 100 μM) for 4, 24 and 48 h. Data are presented as the mean \pm standard deviation of three independent experiments performed in triplicate. * $P < 0.05$ vs. control, # $P < 0.05$ vs. CoNPs group and $\circ P < 0.05$ vs. 10 μM melatonin at same time point. LDH, lactate dehydrogenase; CoNPs, cobalt nanoparticles; mel, melatonin.

Western blot analysis. Western blot analysis was performed to investigate alterations in the expression of proteins associated with apoptosis. The results of the group treated with 100 μM CoNPs and 100 μM melatonin for 24 h revealed that the expression of Bax was decreased and Bcl-2 increased (Fig. 3A), which resulted in a significant reduction in the ratio of Bax/Bcl-2 compared with the CoNP-only treatment group ($P < 0.05$; Fig. 3B). Similarly, significantly reduced expression of cleaved caspase-3 was observed in the 100 μM melatonin treatment compared with the CoNP-only group (Fig. 3C and D). The 10 μM melatonin treatment group exhibited no significant protective effect on the Bax/Bcl-2 ratio or cleaved caspase-3 levels (Fig. 3).

Comet assay. Genotoxicity was observed as cell DNA migrated towards the anode more rapidly following exposure to CoNPs compared with the control. Genotoxicity was revealed by increases in OTM and tail DNA %. Treatment with melatonin (10 and 100 μM) significantly decreased OTM and tail DNA % in the 24 and 48 h groups, and reduced DNA damage was observed when treated with 100 μM melatonin compared with 10 μM melatonin (Fig. 4; $P < 0.05$). However, 4 h coculture demonstrated no significant protective effects.

Immunostaining of γH2AX foci. To obtain increased insight into the potential mechanisms underlying the protective effect of melatonin on CoNP-induced genotoxicity, the presence of DNA-DSBs was determined by γH2AX foci staining. DNA damage increased strongly at 24 h of exposure to 100 μM CoNPs, however, foci counts were decreased at 48 h (Fig. 5). When NRK cells were incubated with CoNPs in the presence of 100 μM melatonin, the indolamine significantly reduced the foci counts compared with the CoNP-only group at 24 and 48 h ($P < 0.05$; Fig. 5). Treatment with 100 μM melatonin for 4 h demonstrated no significant effects on foci counts compared with the CoNP-only treatment group. The effects of 10 μM melatonin on foci counts were markedly lower compared with the 100 μM melatonin treatment group (Fig. 5).

Determination of intracellular ROS content. The effect of melatonin to protect against oxidative stress was investigated

by measuring the levels of intracellular ROS reduction. After NRK cells were exposed to 100 μM CoNPs, the results demonstrated that ROS production significantly increased compared with the control group ($P < 0.05$; Fig. 6). The green color indicates the fluorescence of ROS production (Fig. 6A-D 24 h). Quantitative analysis indicated that ROS production significantly increased to 1.29, 2.07 and 3.78 fold that of controls following exposure to CoNPs for 4, 24 and 48 h, respectively (Fig. 6E-G). However, treatment with melatonin (10 and 100 μM) reduced ROS generation in a concentration-dependent manner compared with the positive control group. Melatonin reduced ROS generation strongly at a maximum of 63% at 48 h (Fig. 6G).

Discussion

A number of studies have been performed to investigate and identify methods of reducing the toxicity induced by CoNPs (26,27). However, to the best of our knowledge, no confirmed solutions have been identified currently. Our previous studies (28,29) indicated that cobalt exposure induced significant cytotoxicity and genotoxicity *in vitro*, and the mechanism may be ROS-dependent (8,12,30). Notably, a previous study demonstrated that metal ions released from nanoparticles lead to less damage compared with nanoparticles (31). Although melatonin has been reported to exert protective effects against oxidative stress, predominantly induced by ions (25,32,33), limited data is available regarding the therapeutic effects of melatonin on the adverse biological reactions associated with CoNPs released from MoM implants. Therefore, the present study investigated the *in vitro* effects of melatonin on CoNP-induced cytotoxicity and genotoxicity in NRK cells.

Reduced viability of CoNP-treated cells compared with control cells observed in the present study may be an indicator for apoptosis. The CCK-8 assay was performed to assess the cytotoxicity of CoNPs, and the results demonstrated a time- and dose-dependent effect of CoNPs in NRK cells compared with controls. Based on the results, the IC_{50} value was calculated and 100 μM CoNPs was used as the positive control for subsequent experiments. Furthermore, 10 μM melatonin was selected as the low concentration and 100 μM melatonin as the high concentration to investigate the effects of this melatonin on CoNP-induced damage. The results of the CCK-8 assay demonstrated that treatment with melatonin reduced CoNP-induced reductions in cell viability. Results of the LDH leakage assay confirmed that melatonin exhibited a protective effect on maintaining membrane integrity.

The ratio of Bax/Bcl-2, which was upregulated in cells exposed to CoNPs in the present study, is an established molecular biomarker associated with the apoptotic mechanism (34). The results of western blot analysis demonstrated that melatonin reduced the ratio of Bax/Bcl-2 compared with the CoNP-only group, which indicates a potential anti-apoptotic function. We hypothesized that the mitochondrial pathway may have a crucial role in the anti-apoptotic effect of melatonin on CoNP-induced apoptosis. Caspase-3 is reported to be essential in the apoptotic pathway (35), and the expression of cleaved caspase-3 was downregulated by melatonin in the present study compared with the CoNP-only

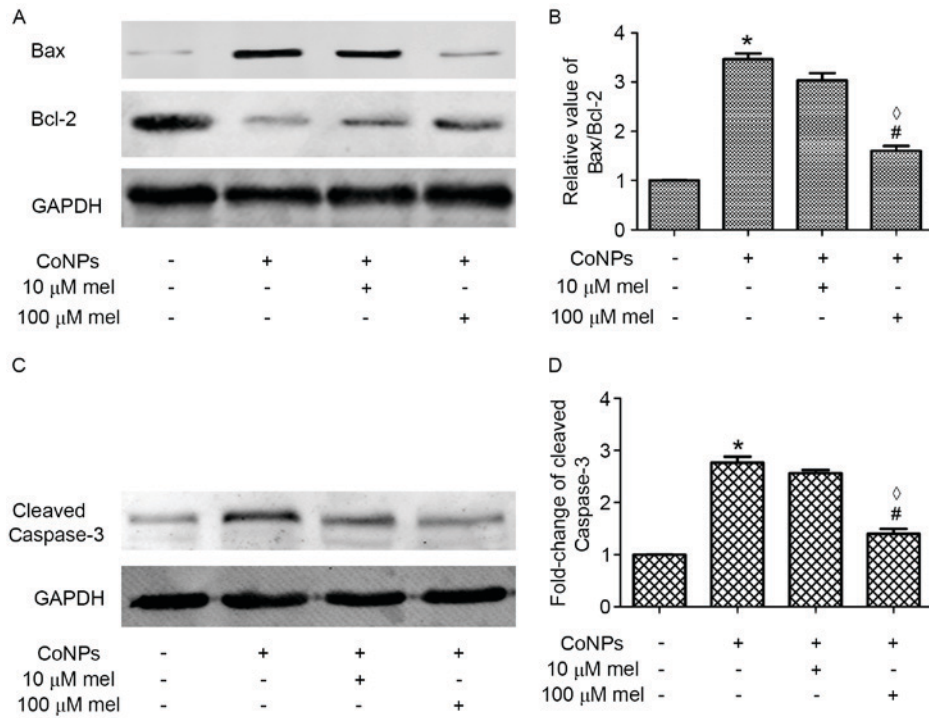


Figure 3. Western blot analysis of apoptosis-associated proteins in NRK cells treated with 100 μM CoNPs and melatonin (10 and 100 μM) for 24 h. GAPDH was used as an internal control. (A) Representative western blot and (B) densitometric analysis of Bax and Bcl-2 protein levels. (C) Representative western blot and (D) densitometric analysis of cleaved caspase-3 protein levels. *P<0.05 vs. control, #P<0.05 vs. CoNPs group and †P<0.05 vs. 10 μM melatonin. CoNPs, cobalt nanoparticles; Bax, Bcl-2-associated X; mel, melatonin.

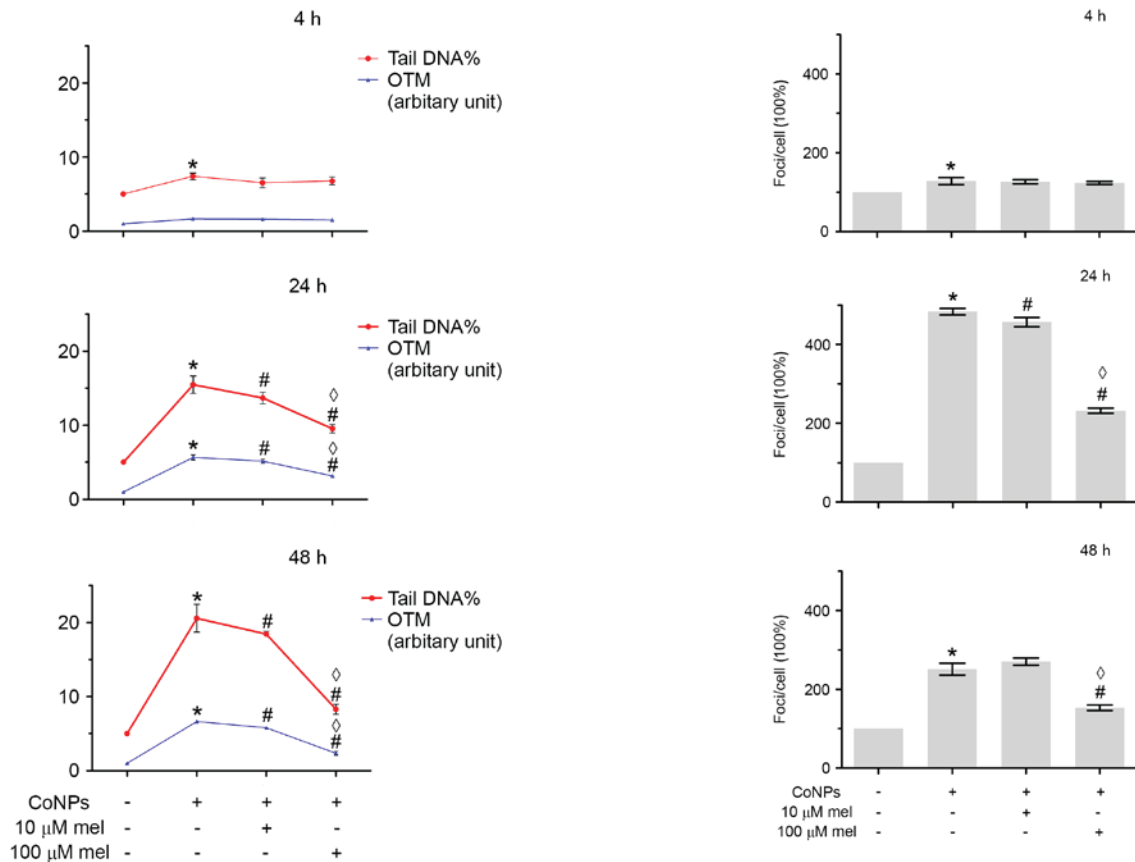


Figure 4. DNA damage was assessed by a comet assay in NRK cells following exposure to 100 μM CoNPs and melatonin (10 and 100 μM) for 4, 24 and 48 h. *P<0.05 vs. control, #P<0.05 vs. CoNPs group and †P<0.05 vs. 10 μM melatonin. CoNPs, cobalt nanoparticles; OTM, olive tail moment; mel, melatonin.

Figure 5. DNA-double strand break analysis following CoNPs exposure. NRK cells were exposed to 100 μM CoNPs and melatonin (10 and 100 μM) for 4, 24 and 48 h. DNA-double strand breaks were quantified by counting the number of phosphorylated-histone 2AX foci per cell using ImageJ software. *P<0.05 vs. control, #P<0.05 vs. CoNPs group and †P<0.05 vs. 10 μM melatonin. CoNPs, cobalt nanoparticles; mel, melatonin.

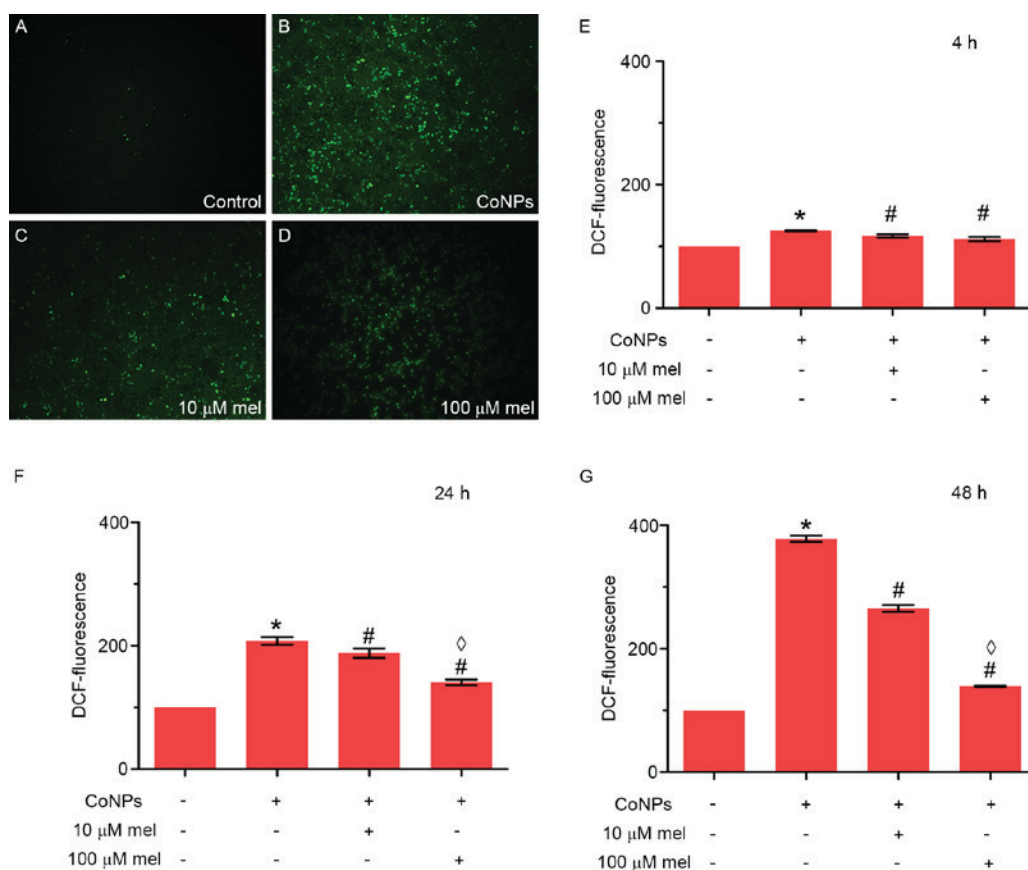


Figure 6. ROS generation following treatment with CoNPs was analyzed by using dichlorofluorescein diacetate. Representative microphotographs (magnification, $\times 40$) demonstrating CoNP-induced ROS generation in NRK cells treated with (A) control, (B) $100 \mu\text{M}$ CoNPs, (C) $100 \mu\text{M}$ CoNPs + $10 \mu\text{M}$ melatonin and (D) $100 \mu\text{M}$ CoNPs + $100 \mu\text{M}$ melatonin (24 h). Quantified results demonstrating the percentage changes in ROS generation in different treatment groups when treated for (E) 4, (F) 24 and (G) 48 h. * $P < 0.05$ vs. control, # $P < 0.05$ vs. CoNPs group and $\diamond P < 0.05$ vs. $10 \mu\text{M}$ melatonin. ROS, reactive oxygen species; CoNPs, cobalt nanoparticles; mel, melatonin; DCF, dichlorofluorescein.

group, which may lead to the inhibition of downstream signaling events and subsequent inhibition of apoptosis induced by CoNPs.

Nanoparticle-induced DNA damage that is initiated by ROS has been previously reported (36), and the results of the comet assay in the current study confirmed that CoNPs are capable of generating ROS, which leads to oxidative DNA damage. Significantly increased levels of DNA damage were observed after 24 and 48 h, and melatonin treated groups significantly reduced DNA damage compared with the CoNP-only group at 24 and 48 h. In order to strengthen these results, the presence of DSBs was analyzed by immunostaining of γH2AX , which is utilized as an early indicator for DSBs and is a highly specific marker. The results indicated that a significant percentage of cells treated with CoNPs exhibited DSBs, consistent with the results of the comet assay. However, after 48 h the foci counts were reduced compared with the 24 h, which indicated that the assay highlighted earlier DNA damage. Furthermore, this phenomenon may also be explained by DNA repair mechanisms and cell death.

Previous studies have indicated that CoNP-induced toxicity is closely associated with oxidative stress (37,38). Intracellular ROS were considered to be the primary factor involved in cytotoxicity and genotoxicity. Generation of excess ROS leads to damage of the mitochondria, lysosomes and the nucleus, and ultimately leads to the activation of

the apoptotic pathway (39). Melatonin and its metabolites have been reported to function as antioxidants (40) and are considered to be efficient free radical scavengers that have the ability to quench a variety of free radicals, including hydrogen peroxide radicals (H_2O_2), superoxide radicals ($\cdot\text{OOH}$) and hydroxyl radicals ($\cdot\text{OH}$). In the present study, CoNPs significantly increased the generation of ROS, while melatonin treatment significantly alleviated this increase. Furthermore, previous studies have demonstrated that the activation of certain antioxidant enzymes may be responsible for the antioxidant effect of melatonin (41,42). These results indicate that melatonin may exhibit a potent antioxidant function, leading to the deactivation of ROS.

The results of the present study indicated that most damage was attenuated when treated with $100 \mu\text{M}$ melatonin after 24 h. Notably, even at a low concentration of melatonin, ROS levels were significantly reduced, which indicated that melatonin exhibits potent antioxidant action. However, the results also indicated that an ROS-independent mechanism may exist, as rapid quenching of free radicals was observed, while cytotoxicity and genotoxicity remained.

Evidence indicates that melatonin acts as an antioxidant against metal ions, exhibiting powerful protective effects against various metal ions, including Al^{3+} , Zn^{2+} , Cu^{2+} , Mn^{2+} , Mg^{2+} and Fe^{2+} (33). However, information regarding the effect of melatonin against nanoparticles is limited. In 1958,

the indolamine melatonin was identified for the first time following purification and identification in bovine serum pineal derivatives by Lerner (43). Subsequent investigation has confirmed its potent antioxidant ability, and, in a variety of free radical-induced diseases, melatonin exhibits beneficial effects in terms of treatment and prevention (40). In addition, melatonin has also been reported to protect against metal-induced oxidative stress due to its free radical scavenging effects, the activation of antioxidant enzymes and metal chelation (33,44). Previous studies have demonstrated that the antioxidant ability of melatonin is superior to other traditional antioxidants, such as vitamin C and vitamin E with limited antioxidant ability, which are unable to provide guidance for clinical medication (19,20). Melatonin exhibits desirable properties, for example, it is ubiquitous, versatile, readily available and nontoxic (45). In the present study, melatonin protected NRK cells from CoNP-induced cytotoxicity and genotoxicity *in vitro* by reducing oxidative stress. These results may contribute to the development of novel strategies to reduce complications associated with nanoparticles released from MoM implants.

In conclusion, the results of the current study have indicated that exposure of NRK cells to CoNPs led to cytotoxicity and genotoxicity, and melatonin ameliorated these effects. These results demonstrate that melatonin may have potential as a therapeutic agent for CoNP-induced cytotoxicity and genotoxicity. The aim of the present study was to elucidate the adverse biological consequences of metal nanoparticles released from hip implants, and provide a potential method for reducing the adverse effects associated with these metal particles.

Acknowledgements

The present study was supported by the Natural Science Foundation of Jiangsu Province (grant no. BK20150399).

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