Bioactive nano-selenium antagonizes cobalt nanoparticle-mediated oxidative stress via the Keap1-Nrf2-ARE signaling pathway

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Research Article

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Abstract

Background

At present, no effective treatment exists for the clinical toxicity of cobalt nanoparticles (CoNPs) after metal-on-metal (MOM) artificial joint replacement. As such, a better understanding of the CoNPs-toxicity mechanism is necessary and urgent for the development of effective and safe detoxification drugs. Our purpose was to explore the role of bioactive nano-selenium (BNS) in antagonizing the toxicity of CoNPs and its mechanism through the Keap1-Nrf2-ARE signaling pathway.

Methods

To examine BNS detoxification, we exposed HUVEC cells to CoNPs (400μmol/l) and BNS (50μg/ml) for 24h, before measuring cell activity, reactive oxygen species (ROS), inflammatory factors, and KNA signaling pathway related transcript and protein expression.

Results

CoNPs stimulate intracellular inflammation and ROS production to bring about significant downregulation of cellular activity. Conversely, BNS reduces ROS generation and suppresses inflammatory factors within cells to reduce CoNPs-mediated cytotoxicity, possibly via the KNA signaling pathway.

Conclusions

Based on our results, BNS antagonizes CoNPs toxic effects by suppressing ROS production through the KNA pathway. Our research provides new insight into the clinical treatment of CoNPs toxicity and explores the potential of BNS in detoxification therapy.

1. Background

Artificial joint replacement is a prosthetic surgery whereby the diseased articular cartilage and bone are replaced with an artificial implant. It has been widely used as an effective treatment for end-stage joint disease[1]. Among the large number of prosthetics available, the metal-on-metal (MOM) artificial joints are the most popular, due to its remarkably high strength and wear resistance[2]. However, due to its high cobalt-chromium alloy content, MOM often releases a large number of cobalt nano particles (CoNPs) into the human body, due to wear (or physical) and chemical factors[3]. Multiple studies have reported CoNPs to be highly toxic. In particular, they have been shown to stimulate intracellular oxidative stress, DNA damage, tissue inflammatory response, and cyto- and genotoxicity[4−5]. Clinically, the CoNPs side effects present as cobalt cardiomyopathy[6], osteolysis around the prosthesis[7], genetic toxicity[8], hearing and vestibular dysfunction[9], blood system diseases like lupus erythematosus and white blood cell abnormalities[10], malignant tumors, inflammatory pseudotumors, and so on. In the skeletal system, for
instance, CoNPs affect the growth and differentiation of osteoblasts, and indirectly up-regulate the dissolution and absorption of bone by osteoclasts, thereby accelerating osteolysis around the prosthesis\[^{11}\]. Based on ample detrimental evidence, the International Agency for Research on Cancer (IARC) listed CoNPs as potentially carcinogenic to humans and graded it as 2B. Our previous research has also confirmed the toxicity of CoNPs on a variety of cells. However, the underlying mechanism still remains to be investigated.

CoNPs are minute particles, averaging a diameter of 50 nm. They are mostly released from the interface wear of artificial joints. Several factors affect the wear of MOM, including implant type, implant position, swing phase load, fluid chemistry, wear process, and isolation technology\[^{12}\]. Moreover, with stress bending, scratching, and impact the surface oxide layer of MOM tends to degrade, thereby releasing CoNPs. In addition, both crevice and galvanic corrosions at the prosthetic joints components, under acidic conditions, further accelerates the release of CoNPs\[^{13}\]. Moreover, CoNPs, that are not absorbed by the bone, can be freely available to multiple organ systems and can exist in forms of particles and corrosion debris, metal-protein complexes, or free metal ions\[^{14}\]. As a result, CoNPs presence can be detected in multiple organ systems like the liver, kidney, pancreas, myocardium, lung, testis, ovary, and so on. Based on published literature, only a small amount of CoNPs can be eliminated from the body through the kidneys\[^{15-17}\].

Reactive oxidative stress (ROS) plays a major role in the toxicity of CoNPs. Once CoNPs enter a cell, they stimulate the production of excessive reactive oxygen species through the Fenton reaction, which in turn, oxidizes cell biomolecules, including DNA, causing DNA strand breakage, and induce a series of other oxidative damage\[^{18}\]. One of the signaling pathways affected by CoNPs is the Keap1-Nrf2-ARE (KNA) antioxidant signaling pathway\[^{19}\]. ROS is known to activate the KNA pathway, which prompts the activation of a large number of downstream antioxidant enzymes, such as heme oxygenase-1 (HO-1), human quinone peroxidase-1 (NQO-1), and so on\[^{20}\]. This is likely the cell’s defense mechanism against the toxicity of CoNPs. As a result, in this study, we suspected that the artificial activation of the KNA pathway to be an effective therapy for CoNPs toxicity.

Bioactive nano selenium (BNS) is a known antioxidant that minimizes oxidative stress in cells\[^{21-23}\]. It is also responsible for generating strong anti-oxidant selenoproteins in the form of selenocysteine, such as GSH, etc. Given its strong antioxidant property, we suspected that BNS mediates its actions via the KNA pathway, and can be used to antagonize CoNPs toxicity\[^{24-25}\]. To test this hypothesis, we evaluated oxidative stress and the underlying mechanism in human umbilical vein endothelial cells (HUVECs), exposed to BNS and/or CoNPs. Based on our results, we have confirmed our hypothesis that BNS protects cells from CoNPs-mediated oxidative stress through the activation of the KNA pathway.

2. Materials And Methods

2.1 Reagents and chemicals
Culture media and solutions like Roswell Park Memorial Institute (RPMI-1640), fetal bovine serum (FBS), penicillin/streptomycin, Trypsin-EDTA, and Phosphate-Buffered Saline (PBS) were purchased from Gibco (Life Technologies, Paisley, UK). CoNPs (30nm, 99.9%) were purchased from Shanghai Chaowei Nano Technology Co., Ltd. (Shanghai, China), BNS (20mg/ml) were sponsored by the Tianjin Orthopaedic Research Institute (Tianjin, China). 10% polyacrylamide gel premix (NCM FastPAGE), RIPA Lysis Buffer, and NcmECL Ultra were purchased from New Cell & Molecular Biotech Co., Ltd (Suzhou, China). Lastly, all antibodies were purchased from Abcam (Cambridge, UK).

2.2 Cell Culture

Human umbilical vein endothelial cells (HUVECs) were purchased from the China Center for Type Culture Collection (CCTCC). The cells were cultured in RPMI-1640 media supplemented with 10% FBS, along with penicillin (100U/ml) and streptomycin (100µg/ml). The medium was replaced daily. All cells were grown in a humidified incubator at 37°C containing 5% CO2. During experimentation, the HUVECs were exposed to specific concentrations of CoNPs (400µmol/l) for 24, 48, and 72 hours to induce CoNPs-mediated toxicity. To test the protective effects of BNS, HUVECs cells were exposed to CoNPs (400µmol/l) and BNS for 24 hours before examining cell viability, ROS production, inflammatory factors, and related protein expression.

2.3 CoNPs preparation

CoNPs were weighed, according to the tested dosages (400µmol/l), sterilized in a high-pressure steam cooker for 4 hours, then suspended in ultrapure water at a concentration of 40 mmol/l, shaken, and mixed in an ultrasonic oscillator for 15 min, before diluting with basic medium (RPMI-1640) to the concentrations used in the experiments. The prepared CoNPs suspensions were stored in a refrigerator at -20°C until experimentation.

2.4 BNS preparation

BNS (20mg/ml), obtained from the Tianjin Institute of Orthopaedics, was irradiated and sterilized under ultraviolet light for 2h, before diluting to 200µg/ml with basal media for subsequent experiments. The diluted BNS was stored at -20°C.

2.5 Cell viability assay

The cell counting kit-8 (CCK-8, Beyotime Company, Jiangsu, China) was used to analyze CoNPs toxicity and BNS detoxification in HUVECs cells. The cells were seeded, at a density of 1×10^4 cells/well in a 96-well plate, using RPMI-1640 with 10% FBS and penicillin (100U/ml) and streptomycin (100µg/ml) and placed in a humidified incubator at 37°C, 5% CO2 for 24 hours. Next, the original medium was replaced with media containing a varying concentration of CoNPs (400µmol/l) or/and BNS (50µg/ml). After 24h, 48h, 72h of incubation with CoNPs or/and BNS, 100µl of CCK-8 solution, diluted to 10% concentration with basic medium (RPMI-1640), were added to the cells. After 2 more h of incubation, the absorbance at 450nm was measured using a microplate reader (Japan Intermed, Tokyo, Japan). Lastly, the relative survival rates of cells, exposed to CoNPs and BNS, for 24h, 48h, and 72h were recorded.
2.6 Cytokine detection

Cells were plated at a density of $1 \times 10^5$ cells/well in six-well plates and incubated for 24 h at 37°C, 5% CO$_2$. Next, CoNPs (400µmol/l) was administered to the cells, with or without the treatment of BNS (50µg/ml), for 24h. Following this, the cell media was analyzed for the presence of secreted inflammatory factors IL-5, IL-8, and TNF-α, using the ELISA kit (R&D Systems, Minneapolis, MN), following manufacturer’s guidelines. Each experiment was repeated 3X.

2.7 Quantification of intracellular ROS

An ROS detection kit (Beyotime Company, Jiangsu, China) and fluorescence imaging were employed to analyze intracellular ROS production in cells exposed to CoNPs and/or BNS. In short, cells were plated at a density of $1 \times 10^5$ cells/well in six-well plates and incubated for 24 h at 37°C, 5% CO$_2$. Then, the original medium was replaced with medium containing CoNPs (400µmol/l) and/or BNS (50µg/ml) for 24h. Next, the cells were incubated with the DCFH-DA from the ROS detection kit for 20 min at 37°C, 5% CO$_2$ before PBS-washing the cells 3X, and observing them under a microscope. The cell morphology and number were recorded under bright field before quantifying the intracellular ROS generation under a fluorescence microscope. The relative values are expressed as a percentage of the fluorescence intensity, as compared to the control.

2.8 Protein analysis using western blot

The cells were plated at a density of $4 \times 10^5$ cells/well in six-well plates and incubated for 24 h at 37°C and 5% CO$_2$. In order to collect all the proteins in the system to ensure the accuracy of the experiment, we collected the cells, cell supernatant and washed PBS. The cells were then exposed to CoNPs (400µmol/l) and BNS (50µg/ml) for 24 h, and the cells media was collected. The remaining cells were once washed with PBS, and the PBS wash was collected. Next, the cells were lysed using RIPA protein lysis buffer (50 mMTris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 0.1% SDS) on ice for 30 minutes, and the resulting cell solution was mixed with the previously stored media and PBS wash. The combined solution was then centrifuged at 12,000×g for 15 min, and the supernatants were recovered. The protein concentration was measured using a spectrophotometer and the reconstituted protein was stored at -80°C until western blot analysis. The western blot analysis was conducted with NCM FastPAGE separating gel and concentrated gel and the resulting membrane was incubated with protein specific primary antibodies (Abcam) overnight. The following day, the membrane was probed with the corresponding secondary antibodies (Abcam) and NcmECL Ultra was used to develop the protein bands before the quantification of Keap1, Nrf2, and NQO1 protein expression.

2.9 Quantitative real-time PCR

Cells were cultured in a 6-well plate at a density of $1 \times 10^6$ cells/well for 24 h before treatment with CoNPs (400µmol/l) with/or BNS (50µg/ml) for a day at 37°C, 5% CO$_2$. Total RNA was extracted from the cells using TRizol reagent, following manufacturer’s guidelines. The cDNA was transcribed from the RNA template using HiScript RT SuperMix (Vazyme, Nanjing, China), then analyzed for the relative expressions
of Nrf2 using AceQ qPCR SYBR Green Master Mix (Vazyme, Nanjing, China) and the MyiO2 Detection System (Bio-Rad Laboratories, Hercules, USA). The primer sequences used for the detection of Nrf2 were as follows: Forward: 5'-ATGCCCTCACCTGCTACTTT-3', reverse: 5'-AGGCCAAGTAGTGTGTCTCC-3'. The expression of each gene was normalized to the endogenous control β-actin, and was expressed, relative to the control, according to the $2^{-\Delta\Delta Ct}$ method.

2.10 Statistical analysis

All data were expressed as mean ± SD (standard deviation) of three independent experiments performed in triplicates. Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by the Dunnet's test. All statistical analyses were performed using SAS 9.4 statistical software (SAS Institute, Cary, NC, USA). A p value less than 0.05 was considered significant.

3. Results

3.1 Optimizing BNS concentration

The cells were incubated with CoNPs (400µmol/l), along with differing concentrations of BNS, for 24h, and the cell viability was assessed using the CCK-8 assay. As shown in Fig. 1, low BNS concentrations, i.e. <45µg/ml, were not able to protect the cells against ROS generation. Similarly, higher concentrations, i.e. >65µg/ml, also did not have a protective effect on the CoNP-exposed cells. Moreover, a concentration of 70µg/ml BNS was found to be toxic for the cells, as evidenced by lower cell viability, as opposed to the CoNPs treatment alone. Based on our results, a BNS range of 45–60µg/ml was shown to be optimal for preserving cell viability, even in the presence of CoNPs, relative to cells exposed to CoNPs alone.

3.2 BNS protects HUVECs from CoNPs toxicity

To quantify the toxicity levels of CoNPs (400µmol/l) in HUVECs, the cells were exposed to CoNPs for 24, 48, and 72 hours (Fig. 2) before measurement of cell viability, via the CCK-8 assay. As shown in Fig. 2A-C, the cell viability decreased over time by 49.96%±3.01%, 49.68%±2.44%, 59.60%±2.73% (p < 0.05) respectively, as compared to cells without CoNPs. Alternately BNS addition antagonized the CoNPs-mediated cytotoxicity and improved cell viability by 37.40%±2.97%, 43.45%±1.93%, 47.17%±2.41% at 24 hours, 48 hours, and 72 hours, respectively (p < 0.05). Moreover, BNS addition alone did not produce any cytotoxicity and the cell viability observed in the BNS-exposed cells was slightly higher than cells that received no treatment.

3.3 BNS exposure suppressed inflammatory cytokines

As shown in Fig. 3, CoNPs exposure significantly upregulated the expression of cytoinflammatory factors IL-1β, IL-6, and TNF-α. Alternately, in cell receiving both CoNPs and BNS, the levels of IL-1β, IL-6, and TNF-α reduced by 42.84% ± 2.16%, 51.05% ± 3.11%, and 43.22% ± 3.46%, respectively (Fig. 3A-C). Moreover, cells receiving BNS alone showed no obvious changes in inflammatory factors and no sign of
cytotoxicity. Taken together, these results suggest that BNS suppresses the CoNPs-mediated activation of inflammatory factors in HUVECs.

### 3.4 BNS antagonizes CoNPs toxicity by activating the KNA signaling pathway

To characterize the underlying mechanism BNS employs to antagonize CoNPs-mediated cell toxicity, we analyzed the expression of the antioxidant signaling pathway KNA-related proteins and its downstream genes (Keap1, Nrf2, NQO1). As shown in Fig. 4 and Fig. 5, cells that received 400µM CoNPs and 50µg BNS for 24 hours, exhibited large increases in the protein Nrf2 and its downstream gene NQO1, as opposed to cells that received CoNPs alone. Based on our results, BNS activated the KNA pathway and its downstream antioxidant proteins to possibly protect the cells against the damaging effects of CoNPs. Notably, we also discovered that CoNPs also activated the KNA pathway. However, this may be the cell's own oxidative stress system gearing up to protect the cell from oxidative damage.

### 3.5 ROS formation

Cells exposed to 400µM CoNPs demonstrated significantly increased ROS levels, as compared to cells with no treatment (Fig. 6). The addition of BNS, however, produced less ROS, as compared to CoNPs treated cells.

### 4. Discussion

Due to the massive integration of artificial joint replacements around the world, more and more artificial joint prostheses are implanted in the human body everyday. Among the available prosthesis, the MOM prosthesis is the most popular due to its unique advantages. However, the MOM prosthesis is not without side effects. Once implanted in the body, nano-scale metal particles are released into the body due to various physical and chemical factors, inducing multiple health complications, including increased blood metal particle concentration, loosening of the prosthesis, pain, and early revision surgery.

Among the many metal particles potentially released by MOM prosthetics, CoNPs are the most toxic. However, not much is known about the underlying mechanism of cyto and genotoxicity caused by CoNPs. One possible mechanism may involve the activation of the intracellular ROS system that produces a large number of ROS system products, thereby increasing intracellular oxidative and gene damage. Oxidative stress often activates the Keap1-Nrf2-ARE signaling pathway, a classic antioxidant pathway that stimulates the dissociation of Nrf2, from its complex with Keap1, so it can travel to the nucleus, where it combines with ARE to activate downstream genes. Due to its anti-oxidative property, KNA pathway is often upregulated when cells are challenged with CoNPs. In this study, demonstrated that BNS can be used to artificially upregulate the KNA pathway to counter the CoNPs-mediated cytotoxicity in HUVEC cells.

Our previous work suggested that the oxidative protection of BNS changes with differing concentrations. In other words, it only protects against toxicity at optimal concentrations. So, we first identified the
optimal concentration of BNS that produces the maximal protection against CoNPs-mediated cell toxicity. We demonstrated that concentrations < 45µg/ml or > 65µg/ml yielded no protection against cytotoxicity. However, the range of 45–60µg/ml BNS was ideal for cell viability, which remained high, as compared to the cells that received CoNPs alone. As a result, all subsequent experimentations involved a BNS concentration of 50µg/ml.

In this study, we used CCK-8 cell viability assay to analyze the rate of cell survival in cells exposed to CoNPs and BNS. Based on our results, any level of CoNPs exposure significantly reduced cellular activity, indicating cytotoxicity. BNS administration, however improved cell viability, even in the presence of toxic CoNPs. Our results were in accordance with others who reported a strong antioxidant property of BNS\cite{31}. Therefore, BNS was used in follow-up examinations to explore the underlying mechanism of cytotoxic protection in conditions of toxic exposure.

ROS is a collective name for a series of naturally occurring oxidative reactive products of oxygen metabolism. However, the presence of certain factors like ultraviolet light and heat exposure sharply increases ROS generation, thereby incurring serious damage to cellular structure and function\cite{32}. For instance, ROS is known to oxidize DNA and induce breakage in DNA strands. Several studies have associated CoNPs to ROS production\cite{4,16}. Similar to other studies, our examinations also revealed that CoNPs-exposed cells had markedly increased ROS production, as opposed to untreated cells. Moreover, the ROS production caused by CoNPs was partly attenuated by the addition of BNS.

Moreover, to characterize the BNS-mediated detoxification pathway, we examined the expression profile of inflammatory factors in CoNPs and/or BNS-exposed cells. Inflammatory factors are often upregulated under conditions of cellular damage and they drive the cell to apoptosis. Oxidative stress is one of the regulatory factors that induce an inflammatory response\cite{33}. Based on our results, CoNPs-exposed cells had remarkably high IL-1β, IL-6, and TNF-α levels, which were partly abrogated in BNS-exposed cells. These results suggest that CoNPs induces oxidative stress in cells and BNS reduces cellular oxidative stress caused by CoNPs.

To further investigate the underlying mechanism of BNS cyto- protection, we explored the role of BNS in the regulation of a well-known antioxidant signaling pathway, KNA. KNA is regulated by cellular oxidative stress and functions by producing a series of antioxidant enzymes. In this study, we demonstrated that BNS administration up-regulated the expression of Nrf2 and its downstream genes, suggesting that BNS activates KNA in order to attenuate oxidative stress, inflammation, cytotoxicity, and possibly genotoxicity. Meanwhile, we also detected activation of the KNA pathway with CoNPs treatment alone. Since oxidative stress is one of the major regulators of the KNA pathway and one of the toxic responses of CoNPs is to elevate ROS production, it is possible that CoNPs activated KNA signaling through its upregulation of oxidative stress.

Our study highlights that CoNPs is highly toxic to HUVECs, and BNS can, in part, abrogate this toxicity. In the presence of BNS, the cells produced less intracellular inflammatory factors and ROS system products.
We also demonstrated that the KNA signaling pathway was employed by BNS in its abrogation of CoNPs cytotoxicity. Our research provides insight into the anti-toxic property of BNS and its underlying mechanism. The information provided in this study can benefit the development of new clinical therapeutics of CoNPs poisoning. Further in-depth study is warranted to determine the role of the KNA pathway in mediating the antioxidant and cytoprotective property of BNS and the specific way in which BNS activates KNA.

Conclusions

Our experiments confirmed that CoNPs have a clear toxic effect, and this effect is mainly achieved by increasing the production of ROS products. Based on this theory, we used BNS as an antidote to study its antagonistic effect and mechanism against the toxicity of CoNPs. The results showed that BNS reduced the inflammatory response and ROS production induced by CoNPs, which was related to the activation of KNA signaling pathway. The existing studies provide a theoretical basis for future in vivo experiments, and help to further study the relationship between KNA signaling pathway and BNS.

Abbreviations

CoNPs: cobalt nanoparticles; MOM: metal-on-metal; ROS: Reactive oxygen species; KNA: Keap1-Nrf2-ARE; IARC: International Agency for Research on Cancer; HO-1: heme oxygenase-1; NQO-1: human quinone peroxidase-1; HUVECs: human umbilical vein endothelial cells; FBS: fetal bovine serum; PBS: Phosphate-Buffered Saline; CCTCC: China Center for Type Culture Collection; CCK-8: Cell counting kit-8; SD: standard deviation

Declarations

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

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

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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

The cell activity of HUVECs after exposure to CoNPs(400μM) and different concentrations of BNS for 24h. All data were expressed as mean ± SD of three independent experiments performed in triplicates. *p<.05 compared with the control group. #p and ##p<.05 compared with the CoNPs group.

Figure 2

The cell activity of HUVECs after exposure to CoNPs(400μM) and BNS(50μg/ml). Comparison of cell viability after exposure to CoNPs and BNS for 24h, 48h and 72h respectively. All data were expressed as mean ± SD of three independent experiments performed in triplicates. *p<.05 compared with the control group. #p<.05 compared with the CoNPs group.

Figure 3

BNS reduced the levels of IL-1β (A), IL-6 (B), and TNF-α (C) induced by CoNPs. Cells exposed to CoNPs (400μM) and BNS (50μg/ml) 24h after the production of cytokine levels. All data were expressed as mean
± SD of three independent experiments performed in triplicates. *p<.05 compared with the control group. #p<.05 compared with the CoNPs group.

Figure 4

The KNA pathway is involved in the process of BNS antagonizing the toxicity of CoNPs. After cells were exposed to CoNPs (400μM) and BNS (50μg) for 24 hours, the expression of Keap1, Nrf2, and NQO1 were detected. Cells cultured with normal medium served as control group.
Figure 5

RT-PCR was performed to detect mRNA expression levels of Nrf2. All data were expressed as mean ± SD of three independent experiments performed in triplicates. *p<.05 compared with the control group. #p<.05 compared with the CoNPs group and control group.
Figure 6

Microphotographs showed generation of cellular ROS in HUVECs exposed to CoNPs and BNS (scale bar, 50 μm). Cells were exposed to 400μM CoNPs with/without BNS for 24h. An upright fluorescence microscope was used to measure the intracellular fluorescence of cells. Each slide was scanned at×100. The green color indicates the fluorescence of detected ROS production. Relative percentage change in ROS generation was measured. Each value represents the mean ± SD of three independent experiments, performed in triplicates.