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ORIGINAL ARTICLE

Cytotoxic, Genotoxic, and Apoptotic Effects of Nickel Oxide Nanoparticles in Intestinal Epithelial Cells

Nikel Oksit Nanopartiküllerinin Bağırsak Epitel Hücreleri Üzerine Sitotoksik, Genotoksik ve Apoptotik Etkileri

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ABSTRACT

Objectives: The superior properties of nickel oxide-nanoparticles (NiO-NPs) have led to their wide use in various fields. However, there is little comprehensive knowledge about their toxicity, especially after oral exposure. The toxic effect of NiO-NPs of mean size 15.0 nm was investigated in Caco-2 (human intestinal epithelial) cells as no study has been performed on their intestinal toxicity.

Materials and Methods: Following identification of their particle size distribution and cellular uptake potential, the risk of exposure to NiO-NPs was evaluated by cellular morphologic changes, cyto- and genotoxic potentials, oxidative damage, and apoptotic induction.

Results: NiO-NPs induced a 50% reduction in cell viability at 351.6 µg/mL and caused DNA damage and oxidative damage at 30-150 µg/mL. It appears that apoptosis might be a main cell death mechanism in NiO-NP-exposed intestinal cells.

Conclusion: NiO-NPs might be hazardous to the gastrointestinal system. The results should raise concerns about using NiO-NPs in food-contact appliances and about NiO-NP-containing wastes. Further *in vivo* and *in vitro* research should be conducted to explain the specific toxicity mechanism of these particles and reduce their risk to humans.

Key words: Nickel oxide nanoparticles, intestinal cells, genotoxicity, oxidative stress, apoptosis

ÖZ

Amaç: Nikel oksit-nanopartikülleri (NiO-NP), üstün özellikleri nedeniyle farklı alanlarda geniş kullanıma sahiptir. Ancak, özellikle oral maruziyete NiO-NP'nin toksisiteleri hakkındaki yeterli bilgi bulunmamaktadır. NiO-NP'nin intestinal sistem üzerine toksik etkisi ile ilgili herhangi bir çalışma bulunmadığından, bu çalışmada NiO-NP'nin (ortalama boyut 15,0 nm) Caco-2 (insan intestinal epiteli) hücreleri üzerine toksisitesi araştırılmıştır.

Gereç ve Yöntemler: Partikül boyutu dağılımı ve hücresel alım potansiyelleri belirlendikten sonar, NiO-NP'ne maruziyerin riski hücresel morfolojik değişiklikler, sito- ve genotoksik etkiler, oksidatif hasar ve apoptoz indüksiyonu ile değerlendirilmiştir.

Bulgular: NiO-NP 351,6 µg/mL konsantrasyonda hücre canlılığında %50 azalmaya ve 30-150 µg/mL'de DNA hasarı ve oksidatif hasarın indüksiyonuna neden olmuştur. NiO-NP'ye maruz bırakılan intestinal hücrelerde ana hücre ölüm mekanizması apoptoz olabilir.

Sonuç: NiO-NP gastrointestinal sisteme tehlikeli olabilir. Elde edilen sonuçlar, gıdayla temas eden cihazların yapımında NiO-NP'nin kullanımı ve NiO-NP içeren atıklar nedeniyle endişeler olabileceğini göstermektedir. NiO-NP'in spesifik toksisite mekanizmalarını aydınlatmak ve insan sağlığı üzerine risklerini azaltmak için daha ileri *in vivo* ve *in vitro* araştırmaların yapılması gerekmektedir.

Anahtar kelimeler: Nikel oksit nanopartikülleri, intestinal hücreler, genotoksisite, oksidatif stres, apoptoz

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INTRODUCTION

Nickel oxide-nanoparticles (NiO-NPs) are widely used as catalysts, pigments, and sensors in different medical and industrial applications because of their physicochemical features.[1,2] NiO and other Ni compounds are regarded as carcinogenic to humans (Group 1).[3] Additionally, NiO-NPs are thought to be more toxic than fine particles are because they have high solubility and release higher amounts of ions in medium.[4] Research has mainly focused on their pulmonary toxicity and reported that the particles induced oxidative stress and inflammatory responses in the airway system.[5-10] NiO-NPs were detected in intestinal tissue after pulmonary exposure.[11] Although it is well known that NiO-NPs could be absorbed through the intestinal tract, there are very few data about the intestinal toxicity of NiO-NPs. NiO-NPs might be harmful to the cells of mucosa.[12,13] On the other hand, the unabsorbed portion is still considered a risk for intestinal cells following oral exposure.

Therefore, in the present study, the toxicity of NiO-NPs was assessed using various endpoints in the Caco-2 human intestine cell line, a highly differentiated human cell line and extensively used to study apical uptake and absorption of nutrients and chemicals as an *in vitro* model for toxicological studies.[14,15] Several studies have also reported the chemical permeability to Caco-2 cells to be correlated well with that of the intestinal membrane *in vivo*.[16]

MATERIALS AND METHODS

NiO-NPs were obtained from Sigma (St. Louis, MO, USA) and recharacterized using transmission electron microscopy (TEM) (JEM-2100 HR, JEOL, USA) and dynamic light scattering (DLS) (ZetaSizer Nano-ZS, Malvern Instruments, Malvern, UK) in distilled water and cell culture medium.[17,18]

Human intestinal epithelial cells (Caco-2, HTB-37) were obtained from the American Type Culture Collection (Gaithersburg, MD, USA). The cells were incubated according to the manufacturer's instructions. Cells at a density of 10^5-10^6 cells/mL were treated with freshly prepared NiO-NPs for 24 h.

To evaluate the cellular uptake potentials of NiO-NPs, inductively coupled plasma-mass spectrometry [(ICP-MS); Thermo Elemental X series 2, USA] was used, while TEM (Jeol-1011, Tokyo, Japan) with an accelerating voltage of 80 kV and an attached digital camera (Olympus-Veleta TEM Camera, Tokyo, Japan) was used to evaluate both cellular uptake and morphology changes at 50 and 100 µg/mL.[17,18]

The cytotoxic activity of NiO-NPs (50-500 µg/mL) was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and neutral red uptake (NRU) assays.[19,20] The optical densities were measured by an enzyme linked immunosorbent assay (ELISA) reader system (Epoch, Germany) at 590 and 540 nm for MTT and NRU, respectively. The enzyme activity inhibition and the accumulation of NR dye in the cells were regarded as cytotoxicity endpoints. The cytotoxicity was calculated compared to that of negative control cells. The median inhibitory concentration (IC_{50}) values were expressed as the concentration of NiO-NPs that induced inhibition of 50% in enzyme activities in cells.

The genotoxic effects of NiO-NPs were determined at concentrations of 15-120 µg/mL by comet assay.[21] Hydrogen peroxide (H_2O_2) (100 µM) was the positive control. Caco-2 cells were mixed with premelted low-melting point agarose, layered on slides previously coated with agarose, covered with a cover slip, and allow to solidify at 4˚C. Then a lysis solution was used to lyse the cells on the slides (for 1-12 h at 4˚C). Electrophoresis was performed for 20 min and the slides were rinsed with neutralization buffer and fixed using 99% ethanol. To score the DNA breaks, ethidium bromide was used to stain the DNA before the examination using a fluorescent microscope supplied with an automated image analysis system (Olympus BX53, Olympus, Tokyo, Japan). The percentage of DNA in the comet tail (tail intensity %) was used to express the DNA damage to individual cells.

Glutathione (GSH), malondialdehyde (MDA), 8-hydroxy-2'-deoxyguanosine (8-OHdG), and protein carbonyl (PC) ELISA oxidative stress determination kits were obtained from Yehua Biological Technology (Shanghai, China), while the dye reagent for the protein assay was purchased from Bio-Rad (Munich, Germany). The oxidative stress parameters in the cells treated with 50-150 µg/mL NiO-NPs were determined according to the manufacturer's instructions and Abudayyak et al.[17] The results were calculated and expressed per gram of protein.

The Annexin V-FITC/propidium iodide (PI) apoptosis/necrosis detection kit was from BioLegend (San Diego, CA, USA). NiO-NP concentrations were 400-700 µg/mL. Based on the manufacturer's instructions, the trypsinized cells were adjusted to be $1x10^6$ cells/mL. Next, 100 µL of cell suspension were mixed with 5 µL of Annexin V-FITC and 10 µL of PI and the resulting mixture was incubated in the dark for 15 min. A phase-contrast fluorescent microscope (Olympus BX53, Olympus, Tokyo, Japan) was used to count the apoptotic and necrotic cells. The percentages of the cells to the total cell amount were expressed as the results of this parameter.[17,18]

Statistical analysis

The cytotoxicity tests were performed in triplicate on four different days (n=12). The other tests were done in triplicate on three different days (n=9). The data were expressed as mean ± standard deviation. The statistical analysis was conducted using One-Way ANOVA Dunnett t-test by SPSS version 23 for Windows (SPSS Inc., Chicago, IL, USA) and p less than 0.05 was selected as the level of significance.

RESULTS AND DISCUSSION

The results of TEM and DLS evaluations obtained from our previous study indicated that the average sizes of NiO-NPs were 15.0 nm (4.2-38.1 nm) in water and 21.4 nm (7.2-60.5 nm) in the cell culture medium. The increase in the medium group might be due the adsorption of medium proteins in the surface of the particles.[17,18]

The results of the cellular uptake by ICP-MS indicate that Caco-2 cells took up NiO-NPs after exposure to 50 and 100 µg/mL for 24 h (Table 1). The agglomeration of NiO-NPs at the high concentration could explain the decreases in their cellular uptake at the high exposure concentration in comparison with the lower exposure concentration.

The NiO-NPs were obtained within the cytoplasmic vacuoles at 50 and 150 µg/mL (NPs shown with red arrows). The particle sizes were larger in the exposed cells at 150 µg/mL compared to those at 50 µg/mL. Moreover, the number of particle-containing vacuoles in the exposed cells decreased when the concentration of NiO-NPs was increased. Cytoplasmic organelles appeared normal and nuclear and plasma membranes were intact in the exposed cells, as in the negative control cells. Electron-dense bodies were visible in the cytoplasm of some cells in both treatment groups. The most notable change in the cells included the presence of electron-lucent large vacuoles filled with NPs. In a few cells at 50 µg/mL, the cytoplasmic vacuoles were so large that they induced disruption of the cytoplasm. In addition, abnormal nuclei with indentations in the membrane and chromatin condensation were seen in some cells in both treatment groups (Figures 1A-1C).

In all the cells, lipid droplets were observed to increase with increasing NP concentrations, which could be an indicator or feature of the oxidative stress process.[22,23] According to TEM pictures and ICP-MS analysis, the NiO-NPs toxicity could be related to the uptake and accumulation of NiO-NPs in the cells.

The results of the cytotoxicity evaluation showed a decrease in cell viability depending on concentration manner. The median IC_{50} values were 479.15 and 351.6 µg/mL by NRU and MTT tests, respectively (Figure 2). According to previous studies, NiO-NPs induced cellular death in different human cell lines such as neuron, liver, lung, airway epithelial, and breast cells and rat kidney epithelial cells.[4,7,23-27] Our results could indicate that the Caco-2 cell line was more vulnerable than other previously studied cell lines to NiO-NP-induced cytotoxic effects.

The genotoxicity of NiO-NPs was estimated using the comet assay (Figure 3). NiO-NPs caused DNA damage (1.2-1.5-fold; $p \leq 0.05$). At 120 µg/mL NiO-NPs, the tail intensity was 8.0%, while it was 13.3% (1.85-fold) in the positive control (100 µM H_2O_2). Previously, researchers showed that NiO-NPs could induce DNA damage in different cells.[28-32] Dumala et al.[12] indicated that NiO-NPs could induce significant DNA damage in the liver, lungs, and kidneys of rats exposed orally to 500 mg/kg bw. Moreover, NiO-NPs caused cell cycle alteration as a consequence of genotoxicity via the nuclear translocation of phosho-ATM and phosphor-ATR in human pulmonary epithelial cell lines.[12] NiO-NPs induced the DNA-damage signaling cascade at 20-100 µg/mL.[18] Li et al.[33] reported DNA damage in pulmonary cells after intratracheal instillation similar to the present results.

Table 1. Nickel oxide-nanoparticles taken up by Caco-2 cells

Exposure concentration (µg/mL/10^5 cells)	Ni amount (mg/10^5 cells)
Negative control	0.19±0.05
50	3.29±0.38
100	1.31±0.22

The assay was performed four times. The results are presented as mean ± SD
Ni: Nickel, SD: Standard deviation

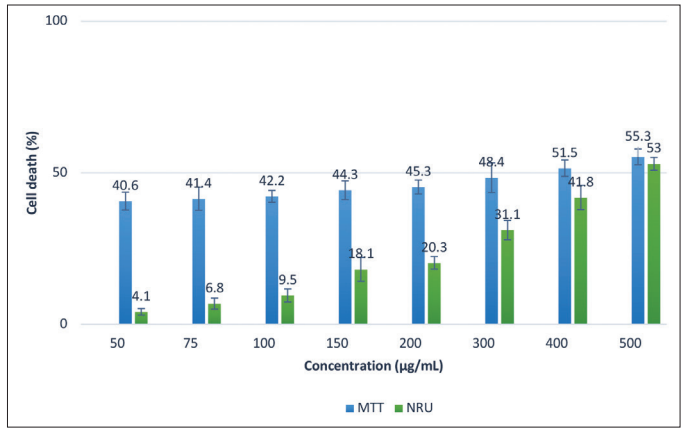

Figure 2. The cytotoxic potential of NiO-NPs

All experiments were done in triplicate and each assay was performed four times. Data are expressed as mean ± SD. The IC_{50} values were 251.6 µg/mL and 479.2 µg/mL in MTT and NRU, respectively
NiO-NPs: Nickel oxide-nanoparticles, SD: Standard deviation, IC_{50}: Inhibitory concentration, MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, NRU: Neutral red uptake

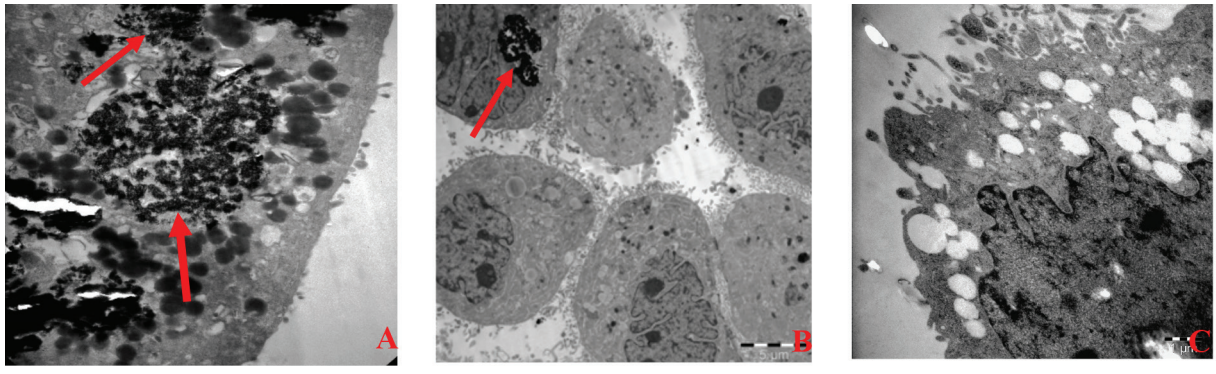

Figure 1. The TEM observations of NiO-NPs uptake by Caco-2 cells

A) Caco-2 cells exposed to NiO-NPs at 50 µg/mL, B) Caco-2 cells exposed to NiO-NPs at 100 µg/mL, C) Caco-2 unexposed cells (negative control)
TEM: Transmission electron microscopy, NiO-NPs: Nickel oxide-nanoparticles

NiO-NP-induced oxidative stress was evaluated with the levels of GSH, MDA, 8-OHdG, and PC (Table 2). NiO-NPs induced oxidative stress as a significant increase in the levels of PC (≤1.5-fold) and a significant decrease in GSH levels (33.1-37.5%) were observed. However, 8-OHdG and MDA levels did not change significantly. In general, the oxidative stress potential of NiO-NPs in Caco-2 cells was statistically significant ($p \leq 0.05$).

The previous data showed that NiO-NPs caused damage in the lung and liver and induce pulmonary inflammation via reactive oxygen species (ROS).[7,8,11,12] *In vitro* research confirmed with *in vivo* studies that NiO-NPs induced an increase in ROS and caused oxidative damage in different human cell lines such as liver, lung, airway epithelial, and breast cells.[24,26,31,34] Previous data confirmed our results; oxidative stress could be the mechanism underlying the cyto- and genotoxicity induced by NiO-NPs.

The Annexin V-FITC/PI assay results demonstrate that NiO-NPs led to induction of apoptosis (Figure 4). The rates of apoptotic cells were 84.6-99.6% of the dead cells, while the rates of necrotic cells were 0.4-15.4% of the dead cells (Figure 4). The results indicated that apoptosis might be the main cell death pathway of NiO-NPs. Di Bucchianico et al.[24] and Saquib et al.[34] observed an increase in the apoptotic cells rate with an increase in NiO-NPs.[24,34] Similarly, Chang et al.[6] indicated endoplasmic reticulum stress-related apoptosis in rats exposed to NiO-NPs by intratracheal instillation.

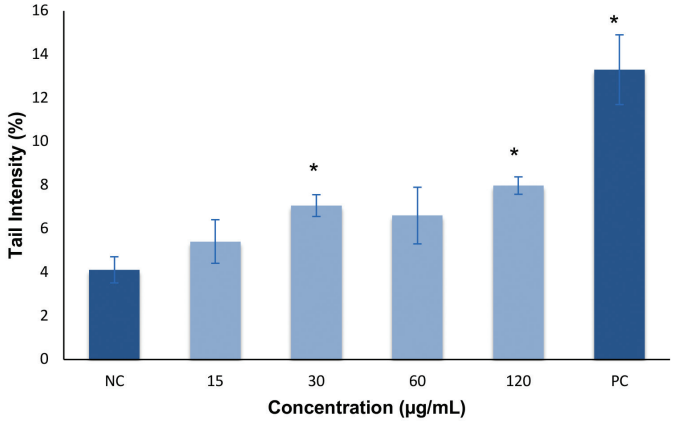

Figure 3. The genotoxic potential of NiO-NPs

All experiments were done in triplicate and each assay was performed three times. The results are presented as mean tail intensity (%) with ± SD. *p≤0.05 was selected as the level of significance by One-Way ANOVA Dunnett t-test
NiO-NPs: Nickel oxide-nanoparticles, PC: Protein carbonyl, SD: Standard deviation

NiO-NPs (25-100 µg/mL) induced apoptosis by functional alterations in mitochondria and lysosomes[30] similar to our results. Previously, changes in mRNA levels in the genes related to the apoptosis pathway were reported.[27,29,34] Duan et al.[35] studied the role of apoptosis in NiO-NP-induced toxicity in human bronchial epithelial cells (BEAS-2B) by investigating the impacts of NiO-NPs on sirtuin 1, a NAD-dependent deacetylase. NiO-NPs (5-20 µg/cm^2) caused cytotoxicity by an apoptotic process, and showed a suppression effect on sirtuin 1, which could underlie the NiO-NP-induced apoptosis via tumor protein p53 and bcl-2-associated X protein. Similar to previous researchers, we observed that NiO-NPs caused dose-dependent apoptosis. The degree of apoptosis/necrosis corresponded with the severity of cytotoxicity (Figures 2 and 4).

CONCLUSION

The toxic effects of NiO-NPs (15.0 nm) were evaluated in the Caco-2 cell line. Our results indicate cellular uptake of NiO-NPs and show cytotoxic potential by disrupting the mitochondrial and lysosomal functions. The median IC_{50} values were 251.6 µg/mL and 479.2 µg/mL by MTT and NRU, respectively. Our results also indicate that apoptosis might be the main cell

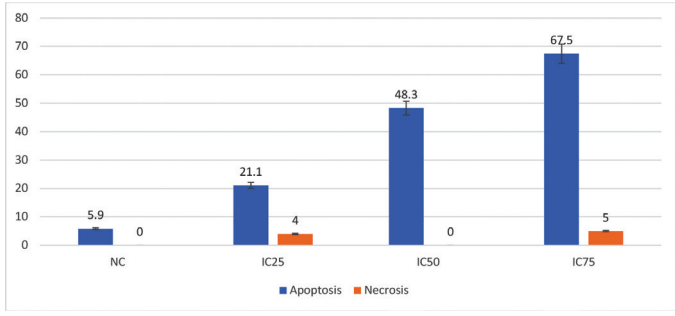

Figure 4. The apoptotic/necrotic potential of NiO-NPs

All experiments were done in triplicate and each assay was performed twice. The results are expressed as the percent of the total cell amount with ± SD, p≤0.05 was selected as the level of significance by One-Way ANOVA Dunnett t-test. Apoptotic ratio was significant at all exposure samples
NiO-NPs: Nickel oxide-nanoparticles, SD: Standard deviation

Table 2. Nickel oxide-nanoparticle induced oxidative damage to Caco-2 cells

Exposure concentration (µg/mL)	8-OHdG (µg/g protein)	MDA (µmol/g protein)	GSH (µmol/g protein)	PC (µg/g protein)
0	0.917±0.132	0.350±0.059	41.142±2.009	5.160±0.108
50	0.964±0.205	0.375±0.108	25.744±3.891*	5.435±0.814
75	0.990±0.242	0.481±0.090	27.500±6.082*	6.667±1.078*
100	1.111±0.310	0.408±0.158	38.092±3.782	7.115±0.877*
150	0.985±0.164	0.442±0.098	39.099±2.82	7.907±1.007*

All experiments were done in triplicate and each assay was performed twice. The results were presented as mean ± SD.
*p≤0.05 was selected as the level of significance compared to the negative controls by One-Way ANOVA Dunnett t-test, MDA: Malondialdehyde, GSH: Glutathione, PC: Protein carbonyl

death pathway after treatment with NiO-NPs for 24 h. Oxidative stress could be the reason for DNA damage induced by NiO-NPs at 15-120 µg/mL concentrations. These results should raise concern about the safety of products that contain NiO-NPs. Further studies should be conducted to elucidate the probable toxicity in the gastrointestinal tract and clarify the pathways of apoptosis and DNA damage.

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