

Assessment of Cellular Responses in Kidney Cells Exposed to Cobalt Oxide Nanoparticles

Mahmoud Abudayyak, Tuba Altınçekiç Gürkaynak, Gül Özhan

ABSTRACT

Cobalt oxide (Co_3O_4) nanoparticles have been extensively used in various industrial and medical applications due to their special optical, magnetic, and electrical activity features. However, there is a lack of information about their toxicity and adverse effects on human health, especially concerning the kidney, which is considered to be a secondary target organ. We investigated the toxic potentials of Co_3O_4 nanoparticles on NRK-52E kidney epithelial cells by *in vitro* assays. Co_3O_4

nanoparticles were taken up by the kidney cells, and caused a decrease in cell viability, by significantly inducing apoptosis/necrosis at 100 $\mu\text{g}/\text{mL}$. However, no significant DNA damage was observed. Co_3O_4 nanoparticles induced cellular toxicity in kidney cells. These results should raise concern about the safety of Co_3O_4 nanoparticles in their various applications. Further studies are needed to elucidate their toxic mechanism.

Keywords: Genotoxicity; Cytotoxicity; Apoptosis; Nanoparticle; Cobalt oxide.

Mahmoud Abudayyak
Istanbul University, Faculty of Pharmacy, Department of Pharmaceutical Toxicology, Beyazıt 34116, Istanbul-Turkey

Tuba Altınçekiç Gürkaynak
Istanbul University, Faculty of Engineering, Department of Chemical Engineering, Avcılar, 34850, Istanbul-Turkey.

Mahmoud Abudayyak, Gül Özhan
Karadeniz Technical University, Faculty of Pharmacy, Department of Pharmaceutical Toxicology, 61000, Trabzon-Turkey (permanent address)

Corresponding Author:

Gül Özhan
e-mail: gulozhan@istanbul.edu.tr

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INTRODUCTION

Nanotechnological products have been used widely in fields ranging from medicine to industry because of their physicochemical properties. However, the human and environmental concern are gradually increased [1]. It is well known that nanoparticles be absorbed through the skin, ingested, and inhaled during occupational and/or environmental applications [2]. With systemic administration, nanoparticles could penetrate from biological membranes. For instance, cobalt (Co)-based nanoparticles easily penetrate and cause damage to the skin more than the bulk material itself [3]. In addition, direct exposure to the bulk materials that are used in various fields can indirectly expose humans to their nanoparticles [4].

Co-based nanoparticles are used in different technological products including sensors, catalysts, pigments, and magnetism and energy storage devices [5-6]. The Co intake in food has been estimated to be 5-40 mg/day [7]. A study that include 970 exposure measurements in Japan reported Co exposures of ambient personal monitoring of 1-6400 mg/m³ [8]. Similar levels were found in the range of 0.9-81 mg/m³ in a German study [9], and levels of 2-240 mg/m³ in a study from the United States [10]. In a Finnish study, 8-hour time-weighted average (TWA) levels were 2-240 mg/m³ [11].

The use of Co is favoured in nanomedicine and nanotechnology due to its enhanced magnetic properties, although it is reported Co_3O_4 is highly toxic and is classified as possibly carcinogenic to humans (Group 2B) by the International Agency for Research on Cancer (IARC) [12-13]. Prolonged exposure of rats and rabbits to Co_3O_4 (0.4-9 mg Co/m^3) resulted in lesions in the alveolar region of the respiratory tract [14]. Also, lifetime exposure of hamsters to Co_3O_4 (7.9 mg Co/m^3) resulted in emphysema [15]. Primary respiratory effects in occupationally exposed humans have been reported as ranging from 0.015 to 0.13 mg Co/m^3 [7]. The no-observed-adverse-effect-level (NOAEL) for chronic inhalation exposure was 0.0053 mg Co/m^3 [7]. The acute median lethal concentration (LC_{50}) for a 30-minute inhalation exposure in rats was 165 mg Co/m^3 [16]. In South Africa, the highest concentrations of cobalt in ambient air and in urine samples of workers were 9.9 mg/ m^3 and 712 $\mu\text{g}/\text{g}$ creatinine, respectively [17]. In previous studies, it has been reported that Co_3O_4 particles were readily taken up through endocytosis and were partially solubilized at the low pH within lysosomes [18-19].

Nanoparticles can affect the cell macromolecules, and have a role in oxidative stress, DNA damage, cell function and morphological change in the exposed organ or system such as lung, liver, kidney, and gastrointestinal and nervous systems [20]. Co_3O_4 , one of the most interesting and widely used Co-NPs, might induce oxidative stress [19], DNA damage and genotoxicity [21-22], cell death, and inflammatory responses [23-25]. It was reported that occupational exposure to Co-NPs has been associated with adverse health effects including rhinitis, asthma, allergic dermatitis, and cardiomyopathy [26]. As the previous studies have been reported, As it is well known, nanoparticle toxicity is still controversial and depends on cell type sensitivity, method and condition of exposure, as well as nanoparticle characterisation [27-28]. In addition, there have been no studies concerning the nephrotoxicity of Co_3O_4 nanoparticles. In this study, we aimed to evaluate by *in vitro* assays the toxic effects of Co_3O_4 nanoparticles on kidney (NRK-52E) cells.

MATERIALS AND METHODS

Chemicals: Co_3O_4 , neutral red dye, triton X-100 and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) were from Sigma Chemical Co. Ltd. (St. Louis, MO, USA). Cell culture medium (DMEM F-12) and all other supplements were from Multicell Wisent (Quebec, Canada). Annexin V-FITC apoptosis (AV) detection kit

with propidium iodide (PI) was from Exbio (Vestec, Czech Republic). The other chemicals were from Merck (NJ, USA).

Particle size characterisation: Co_3O_4 nanoparticles were suspended in milli-Q water and cell culture medium with 10% fetal bovine serum (FBS), and their particle size and distribution were measured by Transmission Electron Microscopy (TEM) (Jem-2100 HR, Jeol, USA) [29-31].

Cell culture and exposure condition: In the study, NRK-52E rat kidney proximal tubular epithelial cells (CRL-1571) was purchased from the American Type Culture Collection (ATCC). The cells were incubated in DMEM-12 medium supplemented with FBS and antibiotics at 5% CO_2 , 90% humidity and 37°C for 24 h. The cell densities were 1×10^6 cells/mL in all assays. Co_3O_4 nanoparticles were freshly suspended at 1 mg/mL concentration in cell culture medium with 10% FBS and sonicated at room temperature for 15 min to avoid the aggregation/agglomeration of the nanoparticles before use [29-31]. The cell exposed final concentrations of 0-750 $\mu\text{g}/\text{mL}$ in the cytotoxicity assays, 0-100 $\mu\text{g}/\text{mL}$ in genotoxicity assay, 0-100 $\mu\text{g}/\text{mL}$ in apoptosis/necrosis assay, and 200 $\mu\text{g}/\text{mL}$ in the cellular uptake assay [30]. The exposure time to the particle suspension was 24 h.

Cellular uptake by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS): To determine cellular uptake of Co_3O_4 nanoparticles, the NRK-52E cells were exposed to 200 $\mu\text{g}/\text{mL}$ concentration of the particle suspensions. The cells were prepared as to Abudayyak et al. [29-30], and counted by Luna cell counter (Virginia, USA). The samples were assayed for Co amount by using ICP-MS (Thermo Elemental Xseries 2, USA). Also, Co content of the untreated cells was measured.

Cytotoxicity evaluation: The cytotoxic potential of Co_3O_4 nanoparticles was determined by MTT and neutral red uptake (NRU) cytotoxicity assays, and AV apoptosis detection assay with PI. By these assays, the induction potential of the particles on metabolism alteration and apoptosis were observed [29-33]. The cell exposed final concentrations of 0, 25, 50, 100, 250, 500 and 750 $\mu\text{g}/\text{mL}$. Optical density (OD) was read by a microplate spectrophotometer system (Epoch, Germany). In every assay, the untreated cells were evaluated as control. It was calculated the inhibition of enzyme activity observed in cells compared to negative control (1% PBS) cells.

To determine the apoptosis induced potentials of Co_3O_4 nanoparticles, it was determined by AV apoptosis detection kit with PI according to supplier instruction. In the assay, it was enabled viable ($\text{AV}^{\text{negative}} / \text{PI}^{\text{negative}}$), apoptotic ($\text{AV}^{\text{positive}}$)

/ PI^{negative}) or (AV^{positive} / PI^{positive}) and necrotic (AV^{negative} / PI^{positive}) cells to be distinguished. The cell exposed final concentrations of 0, 0.1, 10 and 100 µg/mL in the apoptosis/necrosis assay [29-30]. The cells were distributed on the slides and immediately counted under a phase-contrast fluorescent microscope (Olympus BX53, Tokyo, Japan). The cells incubated at 55 °C for 20 min were evaluated as positive control. The untreated cells were used as negative control to define the basal level of the apoptotic cells. The percentage of cells induced to apoptosis was determined by subtracting the percentage of apoptotic cells in the untreated cells from percentage of apoptotic cells in the treated cells. Results were expressed as percent of the total cell amount.

Genotoxicity evaluation: The genotoxic potential of Co₃O₄ nanoparticles was determined by comet assay [29-31, 34]. The cells were exposed to final concentrations of 0, 0.1, 10 and 100 µg/mL. Hydrogen peroxide (H₂O₂) (100 µM) and PBS (1%) were used as positive and negative controls, respectively [29-31]. The number of DNA breaks were scored under a fluorescent microscope (Olympus BX53, Tokyo, Japan) at 400 magnifications by using an automated image analysis system (Comet Assay IV, Perceptive Instruments, Suffolk, UK). DNA damage to individual cells was expressed as a percentage of DNA in the comet tail intensity.

Statistical analysis: All experiments were done in triplicates and each assay was repeated three time. Data was expressed as mean ± standard deviation (SD). The significance of differences between untreated (negative control) and treated cells with nanoparticles was calculated by one-way ANOVA Dunnett t-test using SPSS version 17.0 for Windows (SPSS Inc., Chicago, IL). *p* values of less than 0.05 were selected as the levels of significance.

RESULTS AND DISCUSSION

According to the results of TEM analysis, the average size of Co₃O₄ nanoparticles was 39 ± 21 nm in water with narrow size distribution. When the particle size distribution was evaluated after dispersion in culture medium, the nanoparticles were observed to be slightly agglomerated and/or aggregated in the cell culture medium. Their average sizes (range) were increased to 101.5 nm (32.6-157.1 nm) (Figure 1). With ICP-MS, we observed that Co₃O₄ nanoparticles were taken up by NRK-52E kidney cells. The mean Co amount in the intracellular fluid was 1.5 µg/mL/10⁵ cells. We observed that Co₃O₄ nanoparticles decreased the cell metabolic activity with mitochondrial and lysosomal

dysfunctions with in a concentration dependent manner (Figure 2). The IC₅₀ value of Co₃O₄ nanoparticles on NRK-52E cells was 312.75 µg/mL by MTT assay. According to NRU assay results, the maximum observed cellular death was 21.63% at the maximum exposure concentration of 750 µg/mL. According to these results, we indicate that kidney cells could be a sensitive target due to the cytotoxic effect of Co₃O₄ nanoparticles on mitochondrial function. Alinovi et al. [13] compared the cytotoxic effects of titanium dioxide (TiO₂) and Co₃O₄ nanoparticles on human aortic and umbilical vein endothelial cells. They observed TiO₂ nanoparticles showed few acute cytotoxic effects even at very high concentrations, whereas Co₃O₄ nanoparticles impaired cell metabolism in a concentration- and time-dependent manner. Alarifi et al. [5] reported that Co₃O₄ nanoparticles had a cytotoxic effect on HepG2 liver cells (46.0% cell death at 25 µg/mL for 24 h; 62.0% cell death at 25 µg/mL for 48 h). Similarly, Petrarca et al. [25] observed that Co₃O₄ nanoparticles were cytotoxic on leukemic cancer cells (IC₅₀ value ≤ 21.3 µg/mL).

In the present study, Co₃O₄ nanoparticles induced significant cell death by apoptosis and necrosis on NRK-52E cells (*p* ≤ 0.05). Apoptosis and necrosis frequencies were observed in 70.04% and 29.38% of the dead cells respectively, at an exposure concentration of 100 µg/mL. Co₃O₄ nanoparticles induced apoptosis (≤ 10.64-fold) as well as necrosis (≤ 3.69-fold) in NRK-52E cells compared with negative control cells (Figure 3). Similarly, Co₃O₄ nanoparticles induced apoptosis (at 10-25 µg/mL) in leukemic cancer cells [24]. Spigoni et al. [35] reported that Co₃O₄ nanoparticles significantly reduced cell viability, and induced apoptosis, oxidative stress, caspase activity, and pro-inflammatory cytokine gene expression *in vitro*. They indicated that the adverse effects might be relevant for a potential role of exposure to titanium dioxide (TiO₂) and Co₃O₄ nanoparticles in enhancing cardiovascular risk in humans. Chattopadhyay et al. [24, 28] found that Co₃O₄ nanoparticles significantly induced cell death generated by ROS, which induced tumor necrosis factor-α (TNF-α) by activating pro-apoptotic factors (p38-MAPK, caspase-8, and caspase-3).

In contrast to our results with cytotoxic potential of Co₃O₄ nanoparticles, the particles were observed not to induce DNA damage. No significant differences were found in tail intensity. The tail intensities observed were 16.90 (± 0.74) and 3.71 (± 0.42) for positive (100 µM H₂O₂) and negative control (1% PBS) groups, respectively (Figure 4). However, there are some opposite results in the literature. Alarifi et al. [5] indicated that Co₃O₄ nanoparticles showed a statistically

Figure 1: TEM images of Co_3O_4 nanoparticles in water (a), cell culture medium (b) and the size distributions of Co_3O_4 nanoparticles in water and cell culture medium by TEM analysis (c).

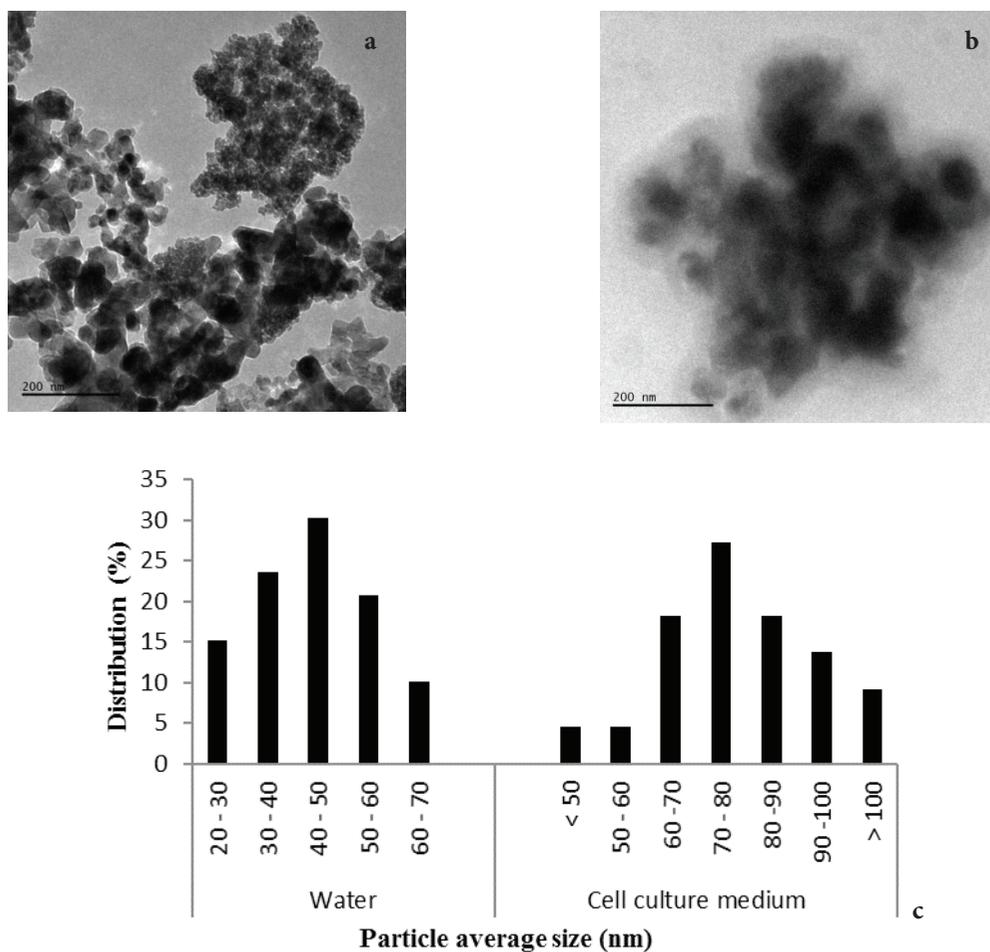
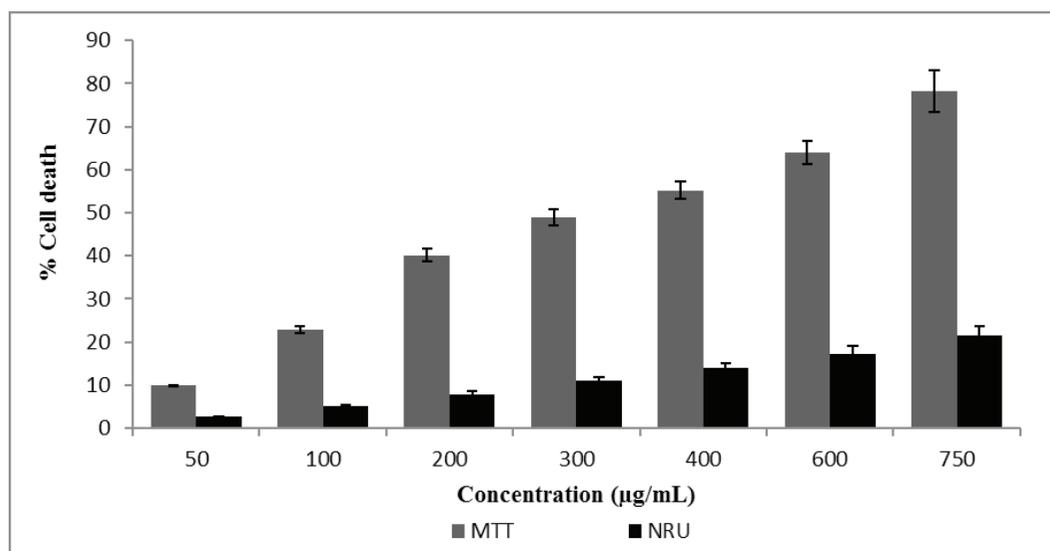
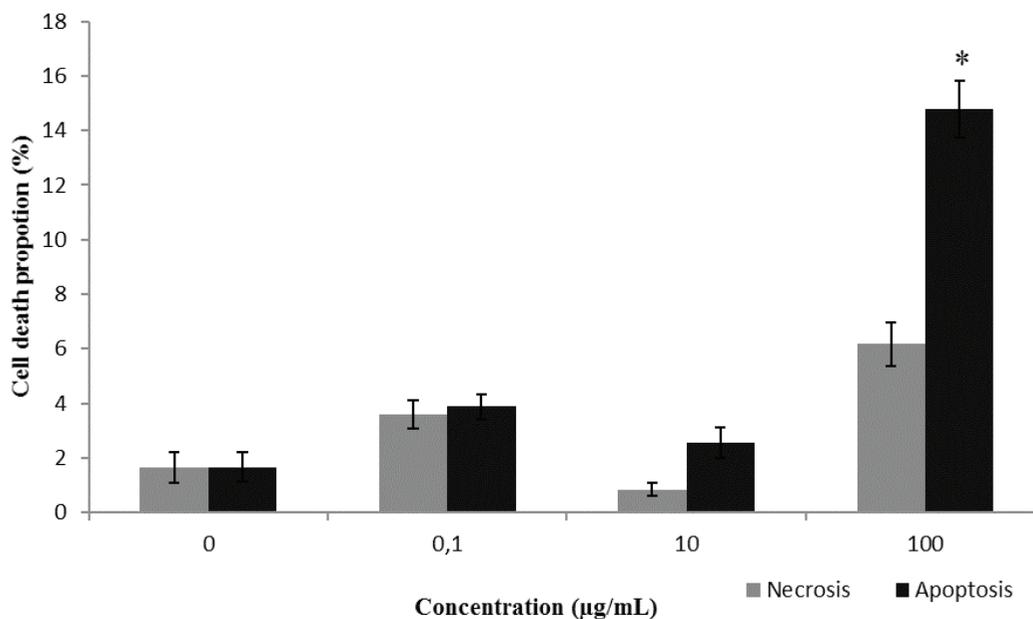


Figure 2: Effect of Co_3O_4 -Nanoparticles on cell viability by MTT and NRU assays.



All experiments were done in triplicates and each assay was repeated four times. The results were presented as mean with \pm SD.

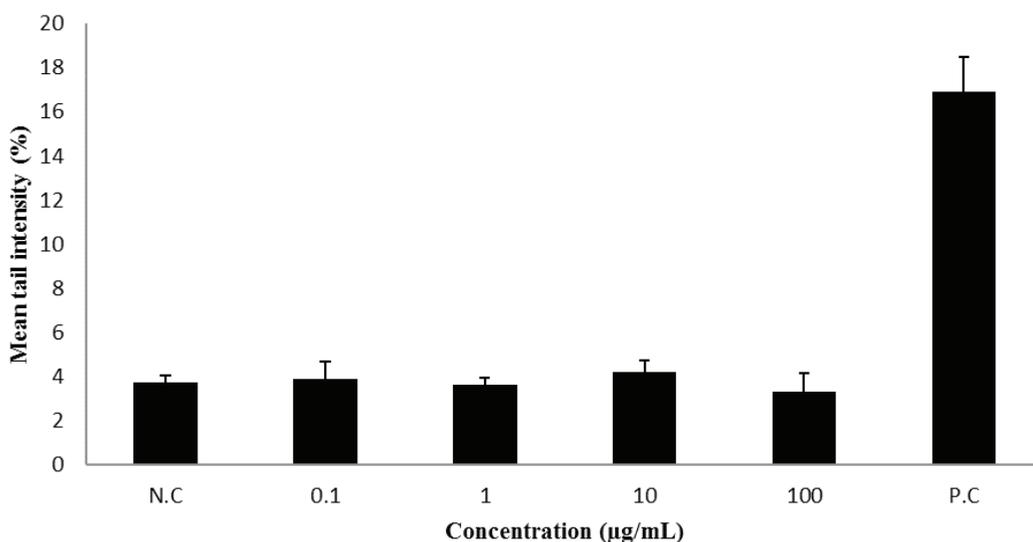
Figure 3: Evaluation on the apoptosis- and necrosis-inducing potentials of Co_3O_4 nanoparticles using AV apoptosis detection assay with PI.



All experiments were done in triplicates and each assay was repeated three times. The results are presented as percentage of the total cell amount with \pm SD.

* $p \leq 0.05$ were selected as the levels of significance by one-way ANOVA Dunnett t-test.

Figure 4: Evaluation of DNA damage potentials of Co_3O_4 nanoparticles using comet assay.



Experiments were done in triplicates and each was repeated three times. The results are presented as mean tail intensity (%) with \pm SD. N.C and P.C are negative and positive controls, respectively.

* $p \leq 0.05$ were selected as the levels of significance by one-way ANOVA Dunnett t-test.

significant dose- and time-related increase in DNA damage. Also, Co₃O₄ nanoparticles caused DNA damage (12.0-24.1 mg/mL) in human leukocytes and chromosomal aberration (100 mg/mL) in human lymphocytes through interacting with DNA and producing reactive oxygen species (ROS) *in vitro* [21, 35]. Alarifi et al. [5] reported that Co₃O₄ nanoparticles caused a significant reduction in GSH with a concomitant increase in lipid hydroperoxide, ROS generation, superoxide dismutase, and catalase activities at 24 and 48 hours. Similarly, Co₃O₄ nanoparticles induced impairment of cellular viability and caused rapid induction of ROS with high levels [19, 36].

Finally, there are few *in vivo* studies about the nephrotoxicity profiles of Co₃O₄ nanoparticles. In rats with 5 mg of metallic Co powder or Co sulphide powder injected into each pole of the right kidney, necropsies were conducted after 12 months, and no tumours were observed in the kidneys of treated or control rats [37]. Rats injected intraperitoneally with Co at 3 or 6 mg/kg body weight exhibited increased levels of oxidatively damaged

DNA bases in the liver, kidney, and lung at 2 and 10 days following injection [38].

In conclusion; we did not notice a correlation between the cell death (apoptosis or necrosis) and the genetic damage. However, we observed Co₃O₄ nanoparticles to be significantly induced cell death, presumably via the other pathways. Co₃O₄ nanoparticles should raise concern about their safety in various applications because the nanoparticles could be possessed of toxicological risk on kidney via cell death, DNA damage and apoptotic effects. However, the results need to be supported with *in vivo* studies to fully understand the mechanism even if the findings are the first results of Co₃O₄ nanoparticle neurotoxicity profiles.

Conflict of interest: Authors declare there is no conflict of interest.

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Kobalt Oksit Nanopartiküllerine Maruz Bırakılan Böbrek Hücrelerindeki Hücresel Cevabın Değerlendirilmesi

ÖZ

Kobalt oksit (Co₃O₄) nanopartikülleri spesifik optik, magnetik ve elektriksel aktivite özelliklerinden dolayı çeşitli endüstriyel ve medikal alanda geniş ölçüde kullanılmaktadır. Ancak, özellikle sekonder hedef organ olarak değerlendirilen böbreklerdeki etkiler göz önüne alındığında toksik ve advers etkileri açısından literatürde yeterli kadar bilgi mevcut değildir. Bu sebeple; Co₃O₄ nanopartiküllerinin NRK-52E böbrek epitel hücreleri üzerinde toksik potansiyelini *in vitro* şartlarda araştırdık. Böbrek hücreleri tarafından alınan Co₃O₄ nanopartiküllerinin 100 µg/

mL maruziyet konsantrasyonunda anlamlı düzeyde apoptoz ve nekrozu indükleyerek hücre canlılığında azalmaya sebep olduğu gözlemlendi. Buna karşılık, DNA'da hasara yol açmadığı tespit edildi. Elde edilen sonuçlara göre; Co₃O₄ nanopartiküllerinin böbrek hücrelerinde hücresel toksisiteyi indüklemesi çeşitli alanlardaki kullanımlarında Co₃O₄ nanopartiküllerinin güvenliliğine temkinli yaklaşılması uygun olacaktır. Ancak, ileri çalışmalar ile toksik etki mekanizmalarının detaylı araştırılması gerekmektedir.

Anahtar Kelimeler: Genotoksosite; Sitotoksosite; Apoptoz; Nanopartikül; Kobalt oksit.

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