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ORIGINAL ARTICLE

In Vitro Evaluation of the Toxicity of Cobalt Ferrite Nanoparticles in Kidney Cell

Kobalt Ferrit Nanopartiküllerinin Böbrek Hücresi Üzerine Güvenliğinin *İn Vitro* Değerlendirmesi

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ABSTRACT

Objectives: The remarkable properties of hard magnetic cobalt ferrite nanoparticles ($CoFe_2O_4$-NPs) and their physicochemical stability lead to various applications in different industrial and medical fields. Although $CoFe_2O_4$-NPs have been reported to cause toxic effects, there is a serious lack of information concerning their effects on the kidneys. In this study, it was aimed to investigate the toxic effects of $CoFe_2O_4$-NPs on NRK-52E kidney cells.

Materials and Methods: The particle characterisation and cellular uptake were determined using transmission electron microscopy, dynamic light scattering and inductively coupled plasma-mass spectrometry. Then, the cytotoxicity was evaluated by MTT and neutral red uptake assays, the genotoxicity by comet assay, and the apoptotic potentials by Annexin V-FITC apoptosis detection assay with propidium iodide.

Results: After 24 h exposure to $CoFe_2O_4$-NPs (39±17 nm), it was observed they did not affect the cell viability at concentration ranging from 100 to 1000 µg/mL, but significantly induced DNA damage at concentration ≤100 µg/mL. No apoptotic or necrotic effect was observed in the exposed cells.

Conclusion: According to the results obtained, $CoFe_2O_4$-NPs are promising for safe use in various applications. However, further *in vivo* studies are needed to fully understand their mechanisms of action.

Key words: DNA damage, cell death, apoptosis, cobalt ferrite nanoparticle

ÖZ

Amaç: Sert manyetik kobalt ferrit nanopartiküllerinin ($CoFe_2O_4$-NP) dikkate değer özellikleri ve fizikokimyasal kararlılıkları farklı endüstri ve tıp alanlarında çeşitli uygulamalarda kullanılmalarına yol açmaktadır. $CoFe_2O_4$-NP'lerin bazı toksik etkilere neden olduğu bildirilmiş olsa da böbrek üzerindeki etkileri hakkında ciddi bilgi eksikliği vardır. Bu çalışmada, $CoFe_2O_4$-NPs'lerinin NRK-52E böbrek hücreleri üzerine toksik etki potansiyellerinin araştırılması amaçlanmıştır.

Gereç ve Yöntemler: Partikül karakterizasyonu ve hücresel alım transmisyon elektron mikroskopu, dinamik ışık saçılma tekniği ve indüktif eşleştirilmiş plazma-kütle spektrometrisi ile gerçekleştirildi. Sonra, sitotoksisite MTT ve nötral kırmızı alım testi, genotoksisite comet tekniği ve apoptotik potansiyel propidyum iyodürlü Annexin V-FITC apoptoz tayini ile değerlendirildi.

Bulgular: $CoFe_2O_4$-NP'lere (39±17 nm) 100-1000 µg/mL arasında değişen konsantrasyonlarda 24 saat süre ile maruz bırakılan böbrek hücrelerinde hücre canlılığının etkilenmediği, ancak ≤100 µg/mL'de önemli ölçüde DNA hasarı meydana geldiği gözlenmiştir. Maruz kalan hücrelerde apoptotik veya nekrotik etki gözlenmedi.

Sonuç: Elde edilen sonuçlara göre, $CoFe_2O_4$-NP'ler çeşitli uygulamalarda güvenli kullanımı vaat etmektedir. Bununla birlikte, etki mekanizmalarının tam olarak anlaşılabilmesi için *in vivo* çalışmalara ihtiyaç vardır.

Anahtar kelimeler: DNA hasarı, hücre ölümü, apoptoz, kobalt ferrit nanopartikülü

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INTRODUCTION

Today nanoparticles are important issue of concern with their widely application in industrial and medical sectors because of their special properties, which cause dramatic increases in intentional and inadvertent oral, dermal and inhalational human exposure. Also, nanoparticles can found as contaminant in water, air, and bulky materials as a result of the natural incident such as volcanic eruptions.[1,2] Research database provides that nanoparticles could cause DNA damage, cell death, oxidative stress and change cell function and morphology *in vitro*, damages and changes in liver, kidney, gastrointestinal and neuronal systems *in vivo*.[3,4]

The exceptional features of cobalt based nanoparticles motivate their uses in different technologies like sensors, catalysts, pigments, and magnetism and energy storage devices.[5,6] Because of the high physicochemical stability of cobalt ferrite nanoparticles ($CoFe_2O_4$-NPs), researchers also focus on using as drug carriers, anticancer treatment, and as magnetic resonance imaging contrast enhancement.[7-9] However, some researchers have shown that $CoFe_2O_4$-NPs could cause oxidative damage, cell death and inflammatory responses in exposed mice, guinea pigs, zebrafish and human cell lines.[10-14] Therewith, both *in vitro* and *in vivo* studies should be gradually carried out to get comprehensive toxicity profiles of nanoparticles to predict their effects on human. There is no study evaluating the effects of $CoFe_2O_4$-NPs or any other cobalt based nanoparticle on kidney. Therefore, we aimed to evaluate the toxic effects of $CoFe_2O_4$-NPs on kidney (NRK-52E) cells by *in vitro* assays.

MATERIALS AND METHODS

$CoFe_2O_4$-NPs (CAT. No: 773352), neutral red dye and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) were obtained from Sigma Chemical Co. Ltd. (St. Louis, MO, USA). Dulbecco's modified eagle medium (DMEM F-12), fetal bovine serum (FBS), phosphate buffered saline (PBS) and antibiotic solutions from Multicell Wisent (Quebec, Canada); Annexin V-FITC apoptosis detection kit with propidium iodide (PI) from Biolegend (San Diego, CA, USA); the other chemicals from Merck (NJ, USA) were purchased.

To particle size and distribution characterization, $CoFe_2O_4$-NPs were suspended in Milli-Q water and cell culture medium with 10% FBS, and measured by transmission electron microscopy (TEM) (Jem-2100 HR, JEOL, USA).[15-17] The average hydrodynamic size of $CoFe_2O_4$-NPs in cell culture medium was determined by dynamic light scattering (DLS) (ZetaSizer Nano-ZS, Malvern Instruments, Malvern, UK). One mg $CoFe_2O_4$-NPs was dispersed in cell culture medium, and then the suspension was sonicated at room temperature for 15 min at 40 W. Ten µL of the suspension were diluted with cell culture medium to reach final concentration 10 µg/mL, and sonicated for further 5 min. Then, DLS experiments performed.

NRK-52E rat kidney proximal tubular epithelial cells (CRL-1571) were obtained from American Type Culture Collection (Rockville, MD, USA). The cells were incubated in DMEM-12 medium supplemented with FBS (%10) and 100 U/mL antibiotic solution at 5% CO_2, 90% humidity and 37˚C for 24 h. The cell densities were from $1x10^4$ to $1x10^6$ cells/mL. $CoFe_2O_4$-NPs 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 exposure.[15,16] The exposure times to the particle suspensions were 24 h.

The cellular uptake of nanoparticle was evaluated with inductively coupled plasma-mass spectrometry (ICP-MS) (Thermo Elemental X series 2, USA). After exposure to 200 µg/mL of nanoparticles, the cells were washed several times with equal volumes of PBS and counted by Luna cell counter (Virginia, USA).[15,16] The acid-digested samples were assayed for Co amount with ICP-MS. Also, Co content of the untreated cells for every cell line was measured.

The cytotoxic potentials of $CoFe_2O_4$-NPs were determined by MTT and neutral red uptake (NRU) assay based on different cellular mechanisms.[15,16,18,19] The cell exposed final concentrations of 0-1000 µg/mL. Optical density was read at 590 and 540 nm for MTT and NRU, respectively, using a microplate spectrophotometer system (Epoch, Germany). In every assay, the untreated cells were evaluated as negative control. It was calculated the inhibition of enzyme activity observed in cells compared with untreated (negative control) cells. Results were expressed as ratio of negative control.

The genotoxic potentials of $CoFe_2O_4$-NPs were determined by comet assay.[15,16,20,21] The cell exposed final concentrations of 0.1-100 µg/mL. Hydrogen peroxide (H_2O_2) (100 µM) and PBS were used as positive and negative controls, respectively. Briefly, the cells were layered on microscope slides coated with agarose gel. The 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), added with 10% DMSO and 1% triton X-100. Then, DNA was unwinded 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). After electrophoresis, slides were neutralized with 0.4 M tris-HCl buffer (pH 7.5) 3 times for 5 min. The number of DNA breaks were scored under a fluorescent microscope (Olympus BX53, Olympus, Tokyo, Japan) at 400 magnification 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 (tail intensity %).

Annexin V-FITC apoptosis detection kit with PI was used to evaluate the cellular apoptosis or necrosis.[15,16] In every assay, negative controls and blank were evaluated. The cell exposed final concentrations of 0.1-100 µg/mL. The apoptotic or necrotic cells, distributed on the slides, were immediately counted at 400 magnification under a phase-contrast fluorescent microscope (Olympus BX53, Olympus, Tokyo, Japan). Results were expressed as percent of the total cell amount.

All experiments were done in triplicates and each assay as repeated four times. Data (n=12) was expressed as mean ± standard deviation. The significance of differences between

the untreated and treated cells with the 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

The aim of this study is to evaluate the toxicity profiles of $CoFe_2O_4$-NPs in NRK-52E kidney cells that could simulate specific target organ or system affected by occupational and environmental exposure to nanoparticles.

According to TEM images, the average size of $CoFe_2O_4$-NPs was 39±17 nm with narrow size distribution after dispersing in water (Figure 1). The nanoparticles slightly agglomerated and/or aggregated after dispersing in the culture medium, and their average sizes (range) increased to 101.5 nm (32.6 to 157.1 nm). The average hydrodynamic size of $CoFe_2O_4$-NPs was evaluated by DLS technique. The nanoparticle size was 183.6 nm (ranging from 5.6-342.1 nm), and 52% of the particles had a size lower than 33.6 nm. In addition, the cellular uptake of $CoFe_2O_4$-NPs was evaluated using ICP-MS. Results confirmed that nanoparticles were taken into the cells. Cobalt concentration was 8.3 µg/mL/10^5 cell compared to the negative control.

In the evaluation of their cytotoxic potential, it was shown that $CoFe_2O_4$-NPs did not decrease the cell viability at concentration ≤1000 µg/mL (Figure 2). Annexin V-FTIC apoptosis detection assay with PI was used to assess the cell death pathway. The maximum levels of apoptotic and necrotic induction were 4.02 and 2.25 fold, respectively. The induction level was statistically significant at 100 µg/mL. Our results showed that apoptosis could be the main cell death pathway in kidney NRK-52E cells exposed to $CoFe_2O_4$-NPs (Figure 3).

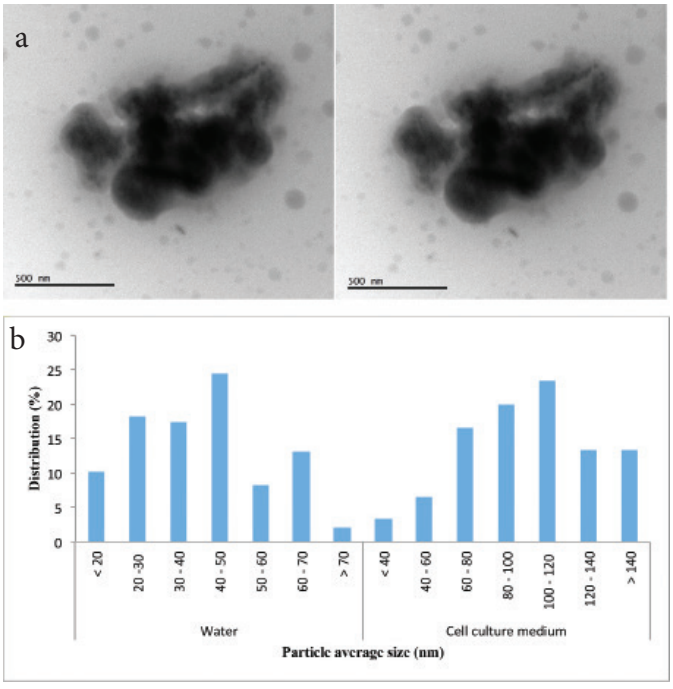

Figure 1. By transmission electron microscopy, the images and a) size distributions of $CoFe_2O_4$-NPs in water and b) cell culture medium

As to Comet assay results, $CoFe_2O_4$-NPs could be genotoxic because it was observed an increase in tail intensity, and induced DNA damage. The increase in DNA damage was significant in the range of 10-100 µg/mL, and occurred in a concentration-dependent manner (p<0.05). At the highest concentration of $CoFe_2O_4$-NPs (100 µg/mL), the tail intensity was approximately 1.7-fold of the negative control. In the positive controls (100 µM H_2O_2), the tail intensity was 16.9 (Figure 4).

DISCUSSION

$CoFe_2O_4$-NPs toxicity still controversial since the previous studies have contrary estimations. Horev-Azaria et al.[13] investigated the *in vitro* toxicological effects of $CoFe_2O_4$-NPs on lung (A549 and NCIH441), liver (HepG2), kidney (MDCK), intestine (Caco-2 TC7), and lymphoblast (TK6) cells in the concentration range of 11.7-281.5 mg/mL. They reported that $CoFe_2O_4$-NPs produced no toxic effects in all cell types at ≤46.9 mg/mL. In that study, a significant decrease in viability was

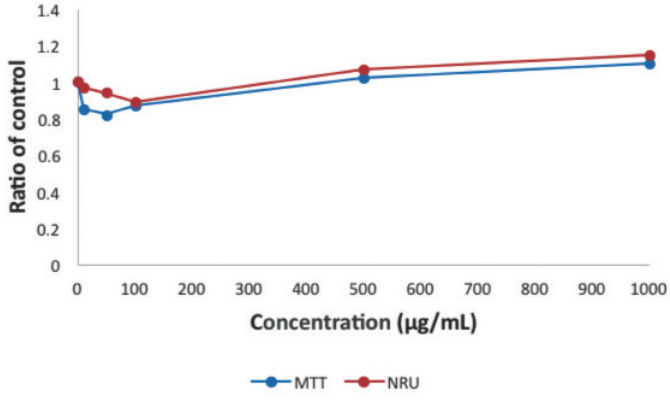

Figure 2. Effects of $CoFe_2O_4$-NPs on cell viability as assayed by MTT and NRU

All experiments were done in triplicates and each assay was repeated four times, The results are expressed as mean

NRU: Neutral red uptake

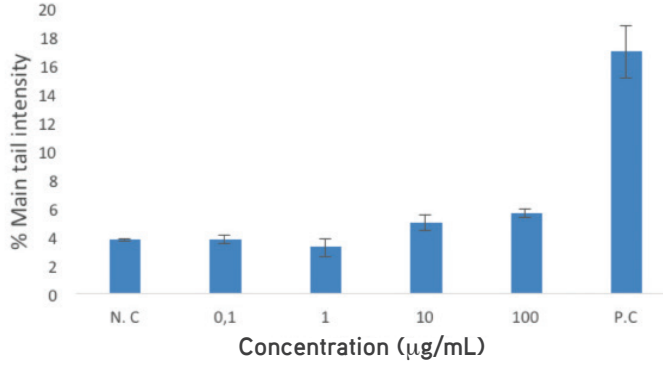

Figure 3. Evaluation of the apoptosis- and necrosis-inducing potentials of $CoFe_2O_4$-NPs as assayed by Annexin V-FTIC apoptosis detection assay with propidium iodide, Results are presented as percentage of the total cell amount, All experiments were done in triplicates and each assay was repeated four times, The results are presented as mean±standard deviation, *T≤0.05 were selected as the levels of significance by one-way ANOVA Dunnett t-test

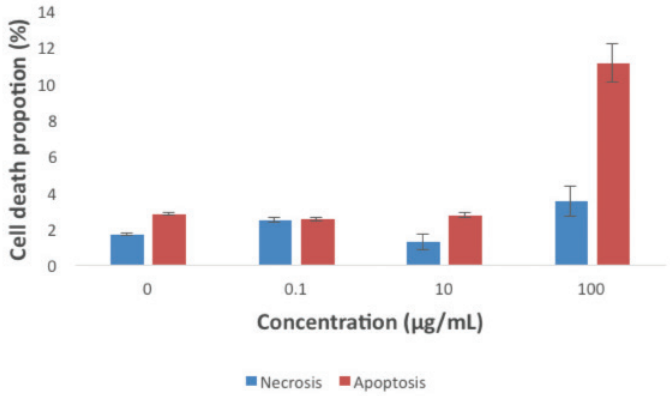

Figure 4. Evaluation of DNA damage potentials of $CoFe_2O_4$-NPs as assayed by comet assay

All experiments were done in triplicates and each assay was repeated four times, The results are presented as mean tail intensity (%) with ±standard deviation, NC and PC mean negative and positive controls, respectively, *p≤0.05 were selected as the levels of significance by one-way ANOVA Dunnett t-test

observed in NCIH441, HepG2, MDCK, and Caco-2 TC7 cells after 72 h, while there was no cytotoxic effect on A549 and TK6 cells even after 24 h of exposure.

Marmorato et al.[22] reported $CoFe_2O_4$ caused interference with lipid metabolism in Balb/3T3 cells depending on concentration. In another study, $CoFe_2O_4$-NPs were observed to have a weakly embryotoxic effect with an IC_{50} value of 243.91 and 20.05 mg/mL in mouse 3T3 fibroblast and D3 embryonic stem cell lines, respectively.[8] Human glioblastoma-astrocytoma (U87MG) cells were observed to have peculiar features including a white corona around the nucleus and other morphological changes after exposure to $CoFe_2O_4$-NPs at 58 and 235 mg/mL for 24 h. They suggested $CoFe_2O_4$-NPs caused cellular stress, and indicated the vesicles appeared to be lipid droplet organelles.[11]

The genotoxicity of $CoFe_2O_4$-NPs was evaluated by studying the interaction with Salmon sperm DNA.[23] It was reported the interaction between $CoFe_2O_4$-NPs and nucleic acid occurred, and the linkage was based on a coordination interaction of the phosphate groups and the oxygen atoms on the heterocyclic bases of DNA with metal ions on the particle surface.[24] Also, Ahmad et al.[10] pointed out the genotoxicity of $CoFe_2O_4$-NPs. Similarly, Colognato et al.[25] reported the induction of genotoxicity in human peripheral lymphocytes exposed those $CoFe_2O_4$-NPs.

CONCLUSION

In conclusion; $CoFe_2O_4$-NPs did not show cytotoxic potentials on the kidney cells, whereas only their highest concentration induced DNA damage. The intensity of toxicological effects of nanoparticles could be varied among different cell lines. In light of the results and previous researches, low but effective concentrations of $CoFe_2O_4$-NPs could be evaluated to be used safely in biomedicine, electronic, magneto-optic, sensor, data storage, catalysis and microwave applications. However, *in vivo* studies should be carried out to fully understand the mechanism of $CoFe_2O_4$-NPs toxicity.

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Conflict of Interest: No conflict of interest was declared by the authors.

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