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Research Article

THE PROTECTIVE ROLE OF OMEGA-3 AGAINST GENOTOXICITY AND REPRODUCTIVE TOXICITY OF COBALT OXIDE NANOPARTICLES ACUTE TREATMENT IN MALE MICE

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ABSTRACT

Objective: Cobalt nanoparticles (NPs), especially cobalt oxide NPs (Co_3O_4 NPs) are attracting unique shaped NPs that are used in different biomedical applications and medicine. Different *in vitro* studies report their toxic and carcinogenic effect but limited *in vivo* studies were present on its genotoxic potential. The present study was aimed to evaluate the genotoxic potential of Co_3O_4 NPs on bone marrow cells and sperms and the protective role of omega-3 in male albino mice.

Methods: Animals were segregated into four groups that were orally treated for 3 consecutive days, Group 1: Negative control; Group 2: Omega-3 (250 mg/kg); Group 3: Co_3O_4 NPs (20 mg/kg); and Group 4: Combined group (250 mg/kg Omega-3 and Co_3O_4 NPs 20 mg/kg).

Results: The present results show that Co_3O_4 NPs administration significantly increased number of micronucleated polychromatic erythrocytes (PCEs)/1000 PCEs, sperm abnormalities, and DNA damage, significantly decreased sperm motility and concentration in comparison to negative control group. However, Omega-3 administration in the combined group modulates the genotoxic potential of Co_3O_4 NPs in comparison to Co_3O_4 NPs group.

Conclusion: The present study reports the genotoxic potential of Co_3O_4 NPs *in vivo* and assesses the protective role of Omega-3 administration due to its antioxidant effect.

Keywords: Co_3O_4 NPs, Omega-3, Micronucleus, Sperm abnormalities, Comet assay.

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INTRODUCTION

Cobalt nanoparticles (NPs), especially cobalt oxide NPs (Co_3O_4 NPs) are attracting enormous interest currently due to their unique shape and size-dependent properties and are used in different biomedical applications and medicine [1,2]. Humans exposed to cobalt NPs from the environment are susceptible to different lung diseases such as fibrosis, interstitial pneumonitis, and asthma [2]. Moreover, IARC [3] evaluated cobalt NPs as a carcinogenic agent.

However, research data on its bioeffects and toxicity are limited. Magaye *et al*. [2] reported the genotoxic potential of cobalt NPs *in vitro*. They reported their induction to malignant mesenchymal tumors in rats. In addition, Bucher *et al*. [4] evaluated carcinogenic potential of cobalt NPs, in which they cause benign lung tumor, bronchioalveolar carcinoma, adenocarcinomas, and bronchioalveolar adenomas in rats that received cobalt NPs for 2 years.

Omega-3 is an essential fatty acid found in large amounts of fish oil. Several researches documented its antioxidant and anti-inflammatory potential [5,6]. It was reported that Omega-3 fatty acids inhibit proliferation, apoptosis, and promote various cancers differentiation [7,8]. Omega-3 fatty acids were found to be hepatic, cardiovascular, and renal protectors; therefore, they have been widely used in clinical perioperative total parenteral nutrition [9,10]. In turn, Omega-3 was determined to be a hopeful protector for different tissue cells against various external toxic stimuli.

Mansara *et al*. [11] reported that Omega-3 supplementation has the ability to significantly ($p<0.01$) increase antioxidant enzymes, superoxide dismutases (SOD), glutathione (GSH) reductase and catalase (CAT) enzymes, activity in red blood cells, and the total plasma antioxidant status in breast cancer patients undergoing chemotherapy. Omega-3 has a protective role against various diseases linked with free radical stress due to its hydroxyl radicals' strong affinity to double bonds that become reduced to single bonds [12].

The present study was aimed to evaluate the genotoxic potential of Co_3O_4 NPs on bone marrow cells and sperms of male albino mice. Moreover, we aimed to estimate the protective role of Omega-3 using cytogenetic and molecular evaluations.

METHODS

Animals

Twelve mature male Swiss albino mice, weighing about 25–30 g b.w. and aged 10–12 weeks were purchased from National Research Center animal house (Dokki, Giza, Egypt). All experimental procedures and animal maintenance were conducted in accordance with the accepted standards of animal care per cage (Council of Europe, European Convention for the Protection of Vertebrate Animals 2006). We have followed the European Community Directive (86/609/EEC) and National Rules on Animal Care.

Tested drugs

1. Co_3O_4 NPs were purchased from Sigma-Aldrich, USA. The product specification from company conforms its structure through X-ray diffraction. It is a black powder with particle size measurements ≤50 nm using transmission electron microscopy photograph. Trace metal analysis is about ≤6000.0 ppm, and its purity meets requirements that reach about 99.5% based on trace metal analysis. They were prepared according to Shaikh and Desai [13], in which Co_3O_4 NPs were suspended directly in 0.9% NaCl saline and then sonicated. It is recommended to keep the suspension in a shaker bath at 30°C during injection to maintain the suspension.
2. Omega-3 (MONTANA) was purchased from Medizen Pharmaceutical Industries, Egypt (therapeutic dose).

Treatment schedule

Mice were segregated into four groups (3 mice/each). All mice were orally administrated according to body weight for 3 consecutive days. Group 1: Negative control group and untreated mice; Group 2: Omega-3 group, mice were treated with Omega-3 (250 mg/kg); Group 3: Co_3O_4 NPs, mice were treated with Co_3O_4 NPs (20 mg/kg); and Group 4: Omega-3 + Co_3O_4 NPs group, mice were administrated orally with Omega-3 (250 mg/kg) simultaneously with Co_3O_4 NPs (20 mg/kg).

Animals were killed by cervical dislocation, then femoral bones and epididymides were used for further assays.

Cytogenetic evaluations

Micronucleus (MN) assay

Bone marrow smears were prepared according to Schmid [14] and then dried smears were fixed in methanol for 5 min and stained with May–Grunwald and Giemsa at pH 6.8.

Semen evaluation of the epididymis

The cauda epididymides were taken from animals and cut into small pieces in 2 mL saline. The sperm concentration and motility rate were determined by adding a small amount of sperm suspension in a hemocytometer counting chambers and examined at high magnification light microscope as described by Watanabe and Endo [15]. To determine sperm abnormalities rate, sperm suspension was smeared on a slide, fixed with methanol for 10 min and stained for 5 mins with 1% eosin, and then washed with water.

Scoring

All glass slides were coded before observation and examined under ×1000 magnification using binuclear microscope. For MN assay: 1000 polychromatic erythrocytes (PCEs) per animal were examined for micronucleated PCEs (MnPCEs).

For semen evaluation: Four large counting chambers were counted and substituted in the following equation to estimate sperm concentration and motility. A total of 1000 sperms were counted to determine the percentage of sperm abnormalities.

The sperm concentration = the total sperm number ÷ $4\times10^4\times2$

The sperm motility rate = the motile sperm number ÷ the total sperm number ×100.

The sperm abnormality rate=the abnormal sperm number ÷ 1000×100.

Molecular evaluation

Comet assay

The alkaline comet assay was performed using bone marrow cells as described in detail by Singh *et al*. [16]. The slides were stained using 80 µL ethidium bromide (20 µg/mL) and then viewed under an epifluorescence microscope (Zeiss epifluoresence) with an attached CCD camera. 50 isolated comets were selected randomly and measured for comet tail length, % DNA in tail and tail moment using COMETSCORE software based on the definition by Olive and Banath [17].

Statistical analysis

Data were expressed as the mean ± standard error (M±SE). Statistical analysis for different assays was performed using student t-test to test the significant difference between groups. All statistics were carried out using statistical analysis systems GraphPad software (GraphPad, 2017)®.

RESULTS

MN assay

Fig. 1a-c shows the induction of Mn in both normochromatic (NCEs) and PCEs erythrocytes due to Co_3O_4 NPs treatment. However, Omega-3 pretreatment in combined group decreases the induction of MnPCEs and MnNCEs as shown in Fig. 1d. The number of MnPCEs/1000 PCEs of mice from Co_3O_4 NPs group significantly increased ($p\leq0.05$) in comparison to negative control group. However, Omega-3 of combined group significantly decreased the induction of MnPCEs/1000 PCEs in comparison to mice from Co_3O_4 NPs group still significant in comparison to negative control group (Fig. 2).

Semen evaluation of the epididymis

Mice treatment with Co_3O_4 NPs (20 mg/kg) induced different sperm abnormalities as shown in Fig. 3. Sperm abnormalities were amorphous, Hookless, banana-shaped, bent and folded heads, bent and folded tails, and bent and looped midpiece. The percentage of sperm abnormalities is significantly higher in comparison to negative control group.

Omega-3 (250 mg/kg) treatment for combined group (Omega-3+ Co_3O_4 NPs) significantly decreased sperm abnormalities percentage in comparison to Co_3O_4 NPs group ($p\leq0.05$) and non-significant to negative control group (Fig. 4).

Moreover, mice from Co_3O_4 NPs group significantly decreased sperm concentration in comparison to negative control group. However, Omega-3 treatment for Omega-3+ Co_3O_4 NPs group significantly increased sperm concentration in comparison to Co_3O_4 NPs group ($p\leq0.05$) and non-significant to negative control group as shown in Fig. 4.

In addition, Fig. 5 shows that Co_3O_4 NPs treatment significantly decreased sperm motility in comparison to negative control group. Omega-3 treatment in the combined group significantly increased sperm motility in comparison to Co_3O_4 NPs group ($p<0.05$) and non-significant to negative control group.

Comet assay

The genotoxic potential of Co_3O_4 NPs was assessed using comet assay, in which Fig. 6a shows typical nuclei of undamaged cells of negative control group; while Fig. 6b represents Omega-3 (250 mg/kg) treated group that shows normal nuclei of undamaged cells; and Fig. 6c represents Co_3O_4 NPs (20 mg/kg) treated group that shows severe DNA damage observed as comets and Fig. 6d shows normal nuclei of undamaged cells of combined (Omega-3+ Co_3O_4 NPs) group. Fifty isolated comets were randomly selected for all groups and measured for comet tail length, % DNA in tail and tail moment using COMETSCORE software. The results show a significant increase in tail length and tail moment

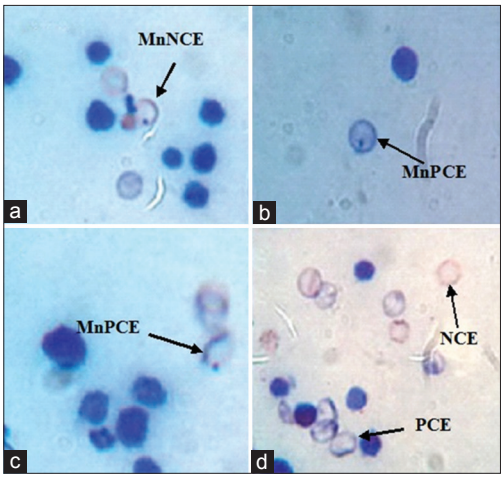

Fig. 1: Representative photomicrographs showing the genotoxic effect of cobalt oxide nanoparticles (20 mg/kg/day) treatment in mice bone marrow cells (a-c) and the protective role of Omega-3 (250 mg/kg) (d). In which, A shows micronucleated normochromatic erythrocytes (MnNCEs), while b-d show MnPCEs with only one micronucleus per cell. PCE represents PCEs and NCE represents normochromatic erythrocytes

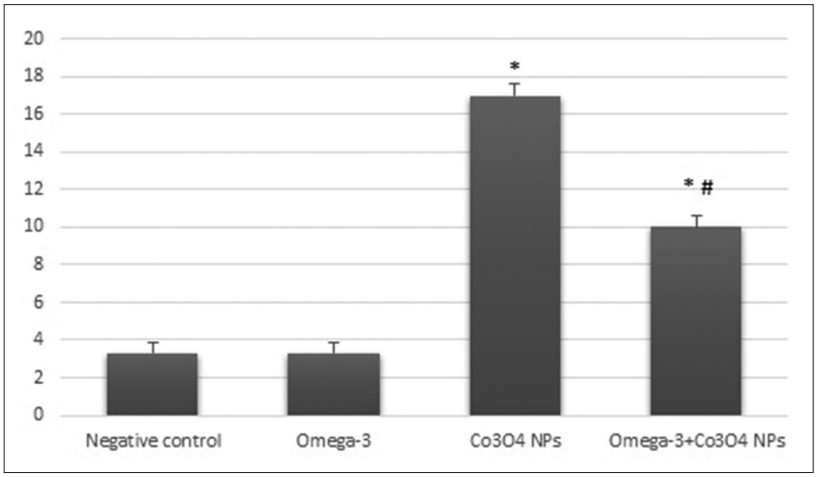

Fig. 2: Effect of cobalt oxide nanoparticles (20 mg/kg/day) treatment and the mitigating role of Omega-3 (250 mg/kg) on the number of micronucleated polychromatic erythrocytes induction in bone marrow cells of mice. Significant difference (p<0.05) between compared groups using student t-test. *Statistically compared with negative control group. #Statistically compared with Co3O4 NP group

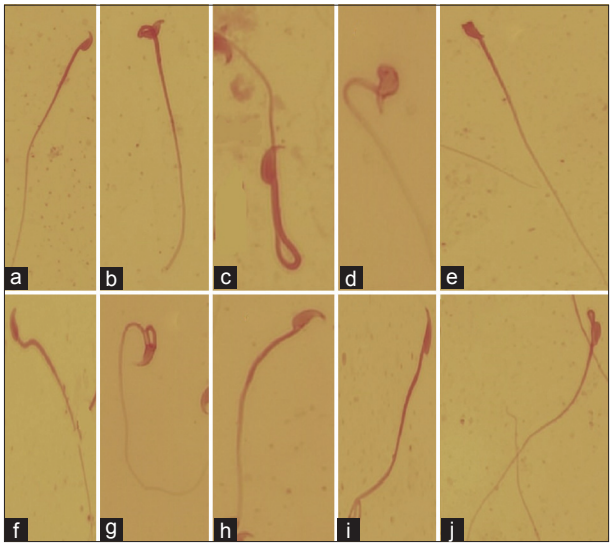

Fig. 3: Representative photomicrograph for the observed normal and abnormal sperms for different untreated and treated mice. In which, (a) represents normal hooked sperm; (b) represents bent and hookless head; (c) represents bent tail; (d) shows amorphous head with a bent midpiece; (e) shows bent head; (f) shows hookless head with a bent midpiece; (g) represents looped midpiece; (h) represents folded tail; (i) shows banana head sperm with a folded tail; and (j) shows sperm with a looped midpiece and hookless head

($p<0.05$) for both Omega-3 and Co_3O_4 NPs groups and % DNA in tail for Co_3O_4 NPs group in comparison with the negative control group (Fig. 7).

Omega-3 treatment of combined group shows a significant decrease ($p<0.05$) of % DNA in tail and tail moment and remarkable decrease of tail length in comparison with Co_3O_4 NPs group. This decrease is non-significant to negative control group except for % DNA in tail.

DISCUSSION

The present study reports the genotoxic potential of Co_3O_4 NPs (20 mg/kg) in bone marrow cells and sperms in male albino mice. Our results were in agreement with several different *in vitro* and few *in vivo* results that estimated the genotoxic effect of cobalt NPs. In which, Ponti *et al*. [18] observed that cobalt NPs (>1 µM) induced an increased production of single- and double-strand DNA breaks as well as chromosomal aberrations in the form of micronucleate binucleate cells in BALB/3T3 mouse fibroblast cells. It was reported that cobalt NPs increased DNA damage by the comet assay and positive results in MN assay in human peripheral leukocytes [19].

However, very few *in vivo* studies were done on cobalt NPs genotoxicity. Klien and Godnić-Cvar [20] believed that the Co NPs have rarely been considered to induce DNA damage *in vivo*. Hereby, we have recorded a significant induction of MN in MnPCEs/1000 PCEs that might be due to chromosomal break/damage leaving a part of chromosome or the whole chromosome in the mature cell [21], reproductive toxicity appeared in the form of decrease in sperm motility, concentration, and increased sperms abnormalities and DNA damage in comet assay in comparison to negative control group. Recently, Hwang *et al*. [22] investigated genotoxicity of Co NPs dependent on surface coating. They reported that silica-coated and uncoated cobalt ferrite accumulated in liver tissue, while only uncoated $CoFe_2O_4$ NPs resulted in enhanced expression of genes related to DNA damage and repair, carcinogenesis, cell death, growth arrest, oxidative stress, and inflammation in mice.

Wise *et al*. [23] suggested that oxidative stress has a playing important role in the toxicity mechanism of different NPs through either the excessive induction of reactive oxygen species (ROS) or depletion of cellular antioxidant capacity, in which induction of free radicals as the superoxide radical (O_2-), H_2O_2, and the hydroxyl radical (·OH) represent the main cause of DNA damage and can leads to cell apoptosis and death [24]. Therefore, ROS induction might be the main reason for Co_3O_4NPs DNA damage that leads to genotoxicity and reproductive toxicity that was explained before by Alarifi *et al*. [25].

Alarifi *et al*. [25] reported that Co_3O_4NPs significantly altered oxidant/antioxidant levels in HepG2 cells due to ROS, lipid peroxidation endpoint product (malondialdehyde, [MDA]), SOD, and CAT activities increased induction, while significantly decreased GSH antioxidant product. They believed that the disturbance of oxidant/antioxidant balance leads to induction of different free radicals O^{2-}, $OH^{\bullet}$, and H_2O_2 that elicit a variety of physiological and cellular events, including inflammation, DNA damage, and apoptosis [26].

Treatment of HepG2 cells with cobalt NPs exposure leads to increase in caspase-3 levels [25] that are essential for cellular DNA damage and apoptosis [27].

Therefore, the main genotoxic target of cobalt NPs could be considered due to oxidative stress that leads to DNA damage and cell apoptosis.

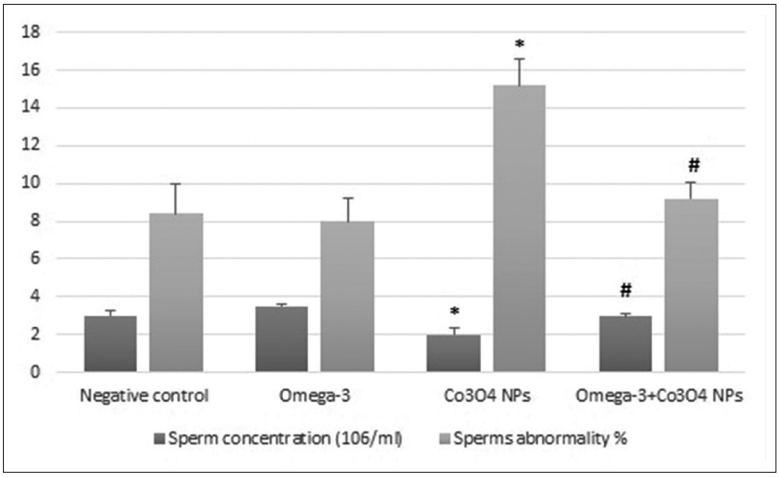

Fig. 4: The genotoxic effect of cobalt oxide nanoparticles (Co_3O_4 NPs) (20 mg/kg) treatment on sperm concentration and the percentage of sperm abnormalities in cauda epididymides of mice. Significant difference (p≤0.05) using student's t-test, in which: *Statistically compared with negative control group; #Statistically compared Omega-3+ Co_3O_4 NPs group with Co_3O_4 NPs group

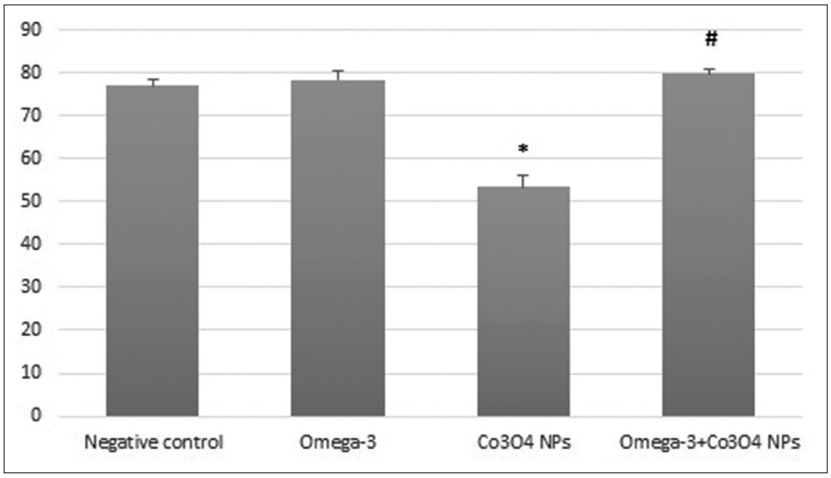

Fig. 5: The effect of cobalt oxide nanoparticles (Co3O4 NPs) (20 mg/kg) treatment on the percentage of sperm motility in cauda epididymides of mice. Significant difference (p≤0.05) using student's t-test, in which: *Statistically compared with negative control group; #Statistically compared Omega-3+ Co3O4 NPs group with Co3O4 NPs group

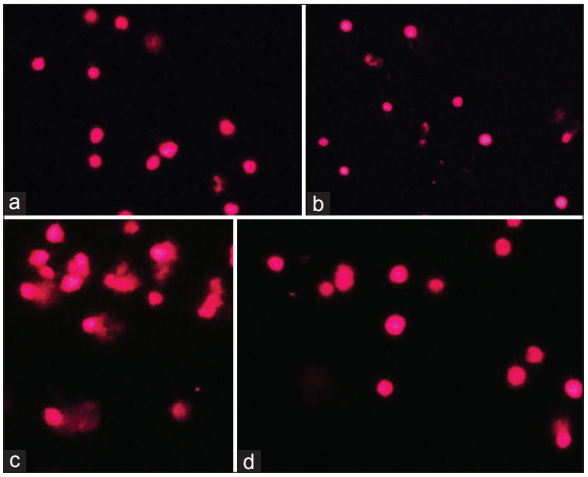

Fig. 6: Representative photomicrograph showing (a) typical nuclei of undamaged cells of negative control group; (b) Omega-3 group; (c) DNA damage observed as comets that were seen in cobalt oxide nanoparticles (Co_3O_4 NPs) group; and (d) combined Omega-3+ Co_3O_4 NPs group

The use of helpful antioxidant could be a good idea to overcome the NP toxicity by its scavenger properties.

The present study reports the protective effect of Omega-3 (250 mg/kg) by modulating the genotoxic potential of Co_3O_4 NPs in the combined group. In which, it significantly decreases the induction of MnPCEs/1000, sperm abnormalities and DNA damage and increases sperm motility and concentration in comparison to Co_3O_4 NPs group. Elelaimy *et al.* [28] elicited the antigenotoxic potential of oral administration of Omega-3 either before or after treatment of the anticancer drug (azathioprine) in male albino mice. In which, they estimated that Omega-3 was effective to reduce the frequencies of MnPCEs, quantitatively decreased the DNA fragmentation as a marker of apoptosis, total sperm different abnormalities and significantly increased sperm number, percentage of PCEs, and enhanced the ratio of PCEs to NCEs (as a marker to cytotoxicity). Moreover, they record the antigenotoxic effect of Omega-3 using polymorphism of DNA (RAPD) assay, that showed distinct differences in animal groups intoxicated with Azathioprine before and after Omega-3 treatment, which reflected DNA protective effect of Omega-3.

These results were in agreement with previous studies, in which Abdou and Hassan [29] reported the protective role of Omega-3 fatty acids

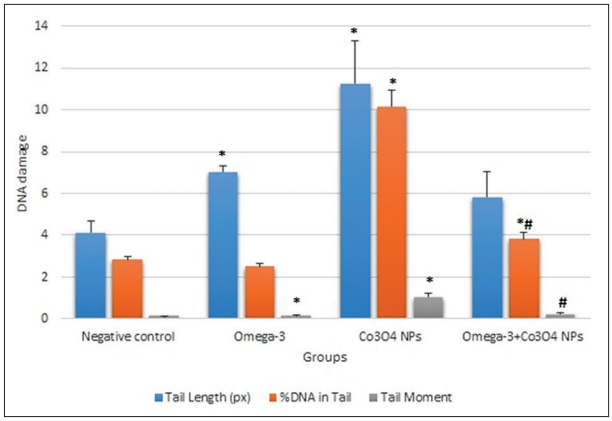

Fig. 7: Effect of Omega-3 (250 mg/kg) on the DNA damage as a result of cobalt oxide nanoparticles (Co_3O_4 NPs) (20 mg/kg) treatment in mice bone marrow cells (represented by comet assay). Significant difference (p<0.05) using student's t-test, in which: *Statistically compared with negative control group; #Statistically compared Omega-3+ Co_3O_4 NPs group with Co_3O_4 NPs group

(125 and 260 mg/kg body weight) in female rats exposed to lead acetate (25 mg/kg body weight). In which, Omega-3 in combined groups with lead acetate normalized the lipid profiles in the serum, hematological parameters, WBCs count and improved the kidney histology to reach the negative control values and architectures.

Recent research evaluated the protective role of fish Omega-3 fatty acids (400 mg/kg, for 30 days by intragastric gavage) on doxorubicin (30 mg/kg, single intraperitoneal injection)-induced testicular apoptosis and oxidative damage in male rats [30]. In addition, Mansara *et al*. [11] estimated in a case report on five Indian women with breast cancer that supplementation of fish oil significantly (p<0.01) increased SOD, GSH reductase, and CAT activity in red blood cells and the total plasma antioxidant status in the patients. They concluded that Omega-3 fatty acids might be an alternative adjuvant treatment for breast cancer instead of chemotherapy due to its antioxidant potential.

Abdou and Hassan [29] indicated that Omega-3 treatment decreased the level of MDA in lead-acetate genotoxic induced groups due to its antioxidant properties to inhibit lipid peroxidation [31] that, in turn, helps to stabilize the reactive radicals and protect cell from damage. Moreover, it was reported that Omega-3 treatment prevented GSH level reduction through protecting the SH group from the reactive free radicals. In addition, it was found that Omega-3 could maintain normal levels of SOD and CAT activities [32]. Therefore, Omega-3, the naturally occurring antioxidant has the ability to scavenge free radical elicited due to Co_3O_4 NPs treatment and, in turn, reduce DNA damage in Comet assay, chromosomal damage in MN assay and reproductive toxicity in sperms.

CONCLUSION

The present study reports the genotoxic potential of Co_3O_4 NPs (Co_3O_4 NPs, 20 mg/kg) in bone marrow cells and reproductive toxicity of sperms in male albino mice. The antioxidant potential of Omega-3 could be the main target to modulate the genotoxic effect induced by Co_3O_4 NPs treatment to maintain the oxidant/antioxidant balance. However, more *in vivo* studies are needed to fully understand the mechanism of cobalt NPs genotoxicity and the antigenotoxic effect of Omega-3.

AUTHORS CONTRIBUTIONS

Nahed A. Hussien contributed in methodology, results interpretation and statistical analysis and wrote the manuscript. Hanan R. H. Mohamed suggested the aim of work, contributed in methodology and revised the final version of manuscript.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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