The Cytotoxicity, DNA Fragmentation, and Decreasing Velocity Induced By Chromium(III) Oxide on Rainbow Trout Spermatozoa

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Abstract

The present study aimed to determine the cytotoxicity of chromium(III) oxide micro particles (Cr2O3-Ps) in rainbow trout (Oncorhynchus mykiss) spermatozoa. Firstly, Cr2O3-Ps were synthesized and structurally characterized the surface, morphological for particle size and thermal properties. In addition, its structural and elemental purity was determined using energy-dispersive X-ray (EDX) spectrum and elemental maps. Structural purity, thermal properties, and stability of Cr2O3-Ps were also examined in detail by performing thermal analysis techniques. The cytotoxicity of Cr2O3-Ps was measured by the observation of velocities, antioxidant activities, and DNA damages in rainbow trout spermatozoa after exposure during 3 h in vitro incubation. The straight line velocity (VSL), the curvilinear velocity (VCL), and the angular path velocity (VAP) of spermatozoa decreased after exposure to Cr2O3-Ps. While the superoxide dismutase (SOD) and the catalase (CAT) decreased, the lipid peroxidation increased in a dose-dependent manner. However, the total glutathione (tGSH) was not affected in this period. DNA damages were also determined in spermatozoa using Comet assay. According to DNA in tail (%) data, DNA damages have been detected with gradually increasing concentrations of Cr2O3-Ps. Furthermore, all of class types which are categorized as the intensity of DNA fragmentation has been observed between 50 and 500 µg/L concentrations of Cr2O3-Ps exposed to rainbow trout spermatozoa. At the end of this study, we determined that the effective concentrations (EC50) were 76.67 µg/L for VSL and 87.77 µg/L for VCL. Finally, these results about Cr2O3-Ps may say to be major risk concentrations over 70 µg/L for fish reproduction in aquatic environments.

Keywords Cr2O3 · Cytotoxicity · Oncorhynchus mykiss · Spermatozoa · DNA damages

Introduction

Recently, although the growth rate in using metal oxide particles has been increased due to their characteristics such as huge specific surface areas, micro interface characteristics, and remediation ability, they can have many potential environmental risks [1, 2]. Metal oxides have been the focus of attention in many studies with their superior electrical properties, thermal stability, and extraordinary morphology [3]. They also have increasing use in many industries such as semiconductors, catalysts, solar panels, and UV protectors. Cr2O3 has a very important place among metal oxides, which has a very wide variety. Besides being used in many electronic applications with approximately 3 eV band spacing, it is used in industrial applications such as paint, pigment, catalyst, solar panels, wear-resistant materials, thermal protection, electrochromic material, and hydrogen storage [4, 5]. This widespread use of Cr2O3 brings some environmental concerns [6, 7].

Anthropogenic sources have many effects that can contaminate aquatic life and these have also toxic agents. However, many toxic contaminants pollute the water system. One of them, chromium (Cr), is a major metallic element posing a maximum threat to all animals and plants according to US-Environmental Protection Agency.
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The main sources of Cr entering the aquatic environment can reach from dyes, mining, electroplating, automobile, and textile manufacturing, metal processing, and leather tanning [8–10]. However, while it is estimated that the production amounts of manufactured metal oxide nano or micro materials in 2020 are 1.663.168 tons [11], the production of Cr in the world is 44,000 gross weight in 2019 [12].

In the modern industry, chromium(III) oxide particles (Cr$_2$O$_3$-Ps) are also used in pigments for ceramics, dyes, paints, and cosmetics [13]. But unfortunately, there is no more information about the toxicological characteristics of this micro material on reproduction system of fish. The US Environmental Protection Agency (EPA) has set a maximum concentration level (MCL) of 100 μg/L for Cr discharged into waters, because it is very toxic [10]. Due to the data that even very small amounts of Cr can be toxic, it became a research curiosity to the interactions in fish reproduction that even very small amounts of Cr can be toxic, it became a research curiosity to the interactions in fish reproduction or aquatic environments in this study.

In a broader context, there are not many studies that addressed the toxicity of Cr$_2$O$_3$-Ps in the literature. In the light of the literature, if we give some examples, researchers studied the lung cells (BEAS-2B) and bronchoalveolar carcinoma-derived cells (A549) of human [14, 15], bacteria in activated sludge system [16], green alga [17], freshwater micro crustacean Daphnia magna and marine bacterium Aliivibrio fischeri [13], Daphnia smilis [18], Escherichia coli [19], Wistar rats [20], and soybean (Glycine max) [21]. In light of these literatures, we could not also find any study about Cr$_2$O$_3$-Ps toxicity on fish or fish reproduction. This study was carried out to determine the cytotoxicity of Cr$_2$O$_3$-Ps on reproduction of rainbow trout (Oncorhynchus mykiss, Walbaum, 1792) with in vitro assay, because it is known that in vitro methods have helped to determine the toxicity levels of environmental pollutants, the mechanisms, exposure times, and concentrations [22]. For this aim, target particles were exposed on the spermatozoa of rainbow trout and it investigated the velocities, movement styles, oxidative stress indices, effective concentrations, and DNA damages of spermatozoa.

Materials and Methods

Chemicals

In this study, the decomposition reaction of ammonium dichromate at high temperatures was used in the synthesis of Cr$_2$O$_3$-Ps. Accordingly, all chemicals used during synthesis and purification were obtained from Sigma-Aldrich. These chemicals were preferred in analytical purity and used without any purification process.

Preparation and Characterization of Cr$_2$O$_3$-Ps

Cr$_2$O$_3$-Ps were prepared by thermal decomposition of ammonium dichromate ((NH$_4$)$_2$Cr$_2$O$_7$) in accordance with the literature [23, 24]. Firstly, (NH$_4$)$_2$Cr$_2$O$_7$ was mixed with ethanol. It was decomposed by combustion reaction in a controlled manner. The degradation of (NH$_4$)$_2$Cr$_2$O$_7$ was initiated at approximately 180 °C and the reaction continued exothermically [25]. When the entire mixture turned dark green, the resulting Cr$_2$O$_3$ was washed with ethanol. It was dried in an oven at 110 °C for 3 h. Obtained Cr$_2$O$_3$-Ps were ground and the chemical structure and purity of the resulting particles were checked by Fourier transform infrared spectroscopy (FTIR), X-ray spectroscopy, and energy-dispersive X-ray spectra. Particle morphology was analyzed by scanning electron microscopy (SEM).

The chemical structures of the obtained Cr$_2$O$_3$-Ps were measured in the range of 400–4000 cm$^{-1}$ using Perkin Elmer Spectrum Two model FTIR device. Crystal property and purity of Cr$_2$O$_3$-Ps were carried out using Rigaku Rad B-Dmax II powder brand X-ray device and were measured in the range of 20–80 2-theta. The morphological properties of Cr$_2$O$_3$-Ps were measured with the LEO EVO-40xVP model SEM device after coating with Au–Pd conductive coating using a Bal-tec brand spatter. Bruker brand Rönteck Xflash EDX detector connected to SEM device was used for elemental verification of Cr$_2$O$_3$-Ps. Particle size analyses of the obtained Cr$_2$O$_3$-Ps were carried out by using Malvern Zetasizer Nano-ZS model device by dispersing it in water. The obtained Cr$_2$O$_3$-Ps thermal properties were determined by differential thermal analysis (DTA) and thermogravimetric analysis (TGA). Shimadzu DTA-50 and Shimadzu TGA-50 devices were used and the analyses were carried out between 30 and 1000 °C using 10 °C/min heating rate in the air atmosphere and used about 10 mg sample in Pt sample pan.

Experimental Design

Semen samples were collected from rainbow trout (Oncorhynchus mykiss, Walbaum, 1792) males (1900 ± 150 g) which were supplied from a commercial farm in Malatya, Turkey, in January 2020. Semen samples were obtained by massage method from the front to the back of the male fish abdomen without anesthesia. The semen pool was taken from 7 individuals. Fresh semen samples in semen pool were diluted with inactivation solution (IS) (NaCl, 103 mM; KCl, 40 mM; CaCl$_2$, 1 mM; MgSO$_4$, 0.8 mM; Hapes, 20 mM (Özgür et al., 2018b); pH 7.8, adjusted with 1 NNaOH) as a stock solution. The pooled sample
was diluted with IS at ratio 1:10 (Semen:IS) to obtain a spermatozoa density of about 12 × 10^8 cells/mL.

In vitro exposure of Cr$_2$O$_3$-Ps was carried out on a semen pool. The semen sub-samples were exposed with nominal concentrations such as 10, 50, 100, 500, and 1000 µg/L of Cr$_2$O$_3$-Ps at 4 °C for 3 h in Eppendorf tubes with the lid closed. The concentrations of Cr$_2$O$_3$-Ps for exposure were determined according to maximum concentration level of chromium that is 100 µg/L in EPA [10]. Semen sub-samples determined according to maximum concentration level of Cr$_2$O$_3$-Ps at 4 °C for 3 h in Eppendorf tubes with the lid closed. The concentrations of Cr$_2$O$_3$-Ps for exposure were determined according to maximum concentration level of chromium that is 100 µg/L in EPA [10]. Semen sub-samples exposed with Cr$_2$O$_3$-Ps at the ratio of 1:20 (chromium:semen with IS) in Eppendorf tubes.

The Velocities and Movement Style of Spermatozoa

The velocities and movement style parameters of spermatozoa were analyzed by the computer-assisted semen analysis system (CASA). The values of velocity parameters such as VSL (straight line velocity, µm/s), VCL (curvilinear velocity, µm/s), VAP (angular path velocity, µm/s), and the movement style parameters of spermatozoa such as LIN (linearity, %, (VSL/VCL)*100), BCF (beat cross frequency, Hz), and ALH (amplitude of lateral displacement of the spermatozoa head, µm) (Özgür et. al., 2019) were examined in the study. For CASA system, model of BASA-Sperm Aqua was used which has an Olympus BX53 microscope (×200 magnification) and a CCD camera (30 fbs) by Merk Biotechnology Ltd. Co. in Turkey. To investigate kinematic parameters under a microscope, the spermatozoa in semen were exposed to activation solution (AS) (1 mM CaCl$_2$; 20 mM Tris, 30 mM Glycine, 125 mM NaCl; pH 9) (Özgür et al., 2018) at ratio 1:20 (Semen:AS). Final dilution level of semen was 2000 times. The EC50 values were calculated by log (agonist) vs. response-variable slope in Graph Prism program.

Oxidative Stress Indices of Spermatozoa

The sperm samples were pooled in a plastic tube to prepare homogenates and 500 µL phosphate-buffered saline (50 mM, pH 7.4) was added to each tube. The homogenization process was performed in cold media; each sample was sonified 6 cycles between each cycle 15 s with an ultrasonifier. Then, the homogenates were centrifuged at 10,000 rpm for 10 min at 4 °C, the supernatants were separated from pellets, and the pellets were stored for malondialdehyde (MDA) analysis. In the beginning, to estimate the protein level in the supernatants, the Bradford method was applied and bovine serum albumin (BSA) was used as a standard [26]. After that, antioxidant enzymes were assayed. For instance, CAT activity was measured spectrophotometrically by following the decrease in the absorbance of hydrogen peroxide (H$_2$O$_2$) at 240 nm at room temperature [27]. One unit of CAT activity is defined as the amount of enzyme required to decompose 1 micromole of H$_2$O$_2$ per minute. SOD activity was measured by using the xanthine oxidase/cytochrome C method reported by McCord and Fridovich [28]. Absorbance was measured at 550 nm on a Shimadzu 1601 UV-Vis spectrophotometer. One unit of SOD activity is the amount of the enzyme required to cause a half-maximal inhibition of cytochrome C reduction. Besides, tGSH was obtained spectrophotometrically by using previously reported method [29]. The colorimetric assay was followed the increase in the formation of 5-thio-2-nitrobenzoate (TNB) which was measured spectrophotometrically at 412 nm. MDA is a biomarker for lipid peroxidation. The MDA level of samples was assayed spectrophotometrically at 532 nm via the method described by Buege and Aust [30]. CAT and SOD levels were expressed as U/mg protein, while the MDA and tGSH results were presented as nmol/mg protein. Six independent samples were used for all measurements.

The Analysis of DNA Fragmentation in Spermatozoa Using Comet Assay

The comet assay was performed under alkaline condition. During the experiment, a version adapted to spermatozoa in study of Singh et al. (1988) was utilized [31]. Previously chilled microscope slides were dipped into 0.7% extremely hot (approximately 70 °C) normal melting agarose prepared phosphate buffered saline (PBS) solution. Afterwards, 10 µL (the final concentration of the spermatozoa on our slides was ~100,000 cells/mL) of previously diluted (1:100) spermatozoa was mixed with 90 µL low melting point agarose (LMA, prepared as 0.5% in PBS), and then, 70 µL of the mixture was added onto the prepared normal melting agarose-covered microscope slides as a top layer. The slides including negative control (just spermatozoa) and positive control (spermatozoa treated with 35% w/w of hydrogen peroxide, the last concentration: 200 µM) were put on a frozen flat tray for 10 min to solidify. Before using ice-cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris base, pH 10), 1% Triton X-100 and 10% DMSO were added into the solution. Prepared slides were then cautiously immersed in lysis solution for at least 1 h at 4 °C. Slides were then removed from the lysis buffer and placed in an electrophoresis tank. The electrophoresis tank was filled with freshly prepared electrophoresis buffer (0.3 M NaOH, 1 mM EDTA, pH 13). Before electrophoresis, the slides were left in the electrophoresis solution for 30 min to allow unwinding of DNA. After this procedure, electrophoresis system was turned on for 30 min at 300 mA and 15 V. After electrophoresis, slides were taken from the horizontal tank and washed three times with 0.4 M Tris buffer, pH 7.5 for 5 min to neutralize the alkali condition. For each slide, 60 µL of ethidium bromide (EtBr concentration
10 mg/mL) was pipetted into the sample. Then, slides were covered with a coverslip and taken images using the inverted microscope (Olympus CKX41) with a combined fluorescence system at ×40 magnification with Olympus cellSens Entry 2.1 software. Comets are formed upon the principle of releasing damaged DNA from the core of the nucleus during electrophoresis. In terms of tail length, tail and head intensities, and tail and head DNA percentages, 100 cells per sample (two duplicate sample slides, 50 randomly selected cells scored per slide) were scored using image analysis software CometScore 2.0. Totally, 100 cells were scored per slide in duplicate. Analysis was performed blindly by one slide reader. DNA fragmentation/damage scoring is visually classified based on the size of the comet tail: class 0 (undamaged-intact DNA); 1, 2, 3, and 4 classes of damages meaning from the less to the most fragmented DNA.

Statistics Analysis

Normality test and descriptive analysis (means ± SE, \( p < 0.05 \)) were performed between the data in the SPSS 15 program. The differences between groups were done by variance analysis (one-way ANOVA) with the Tukey test after the test of homogeneity of variance in each group. Graph Pad Prism 5 for drawing graphics was used.

For DNA damages/fragmentation analysis in spermatozoa using Comet assay, the compliance of data to normal distribution was examined with Kolmogorov–Smirnov test. The data were summarized with median, minimum, and maximum values. Since all groups did not conform to normal distribution, Kruskal–Wallis test and then Conover pairwise comparison method was used for comparisons. The significance level was accepted as 0.05 and 0.001 tails and head of spermatozoa. Moreover, a biostatistics web application was also used for the boxplot graphs (Arslan et. al., 2018).

Results

Characterization of \( \text{Cr}_2\text{O}_3 \)-Ps

FTIR spectrum of \( \text{Cr}_2\text{O}_3 \)-Ps is given in Fig. 1a. Three main regions in the FTIR spectrum of \( \text{Cr}_2\text{O}_3 \)-Ps have been clearly seen. The first region between 400 and 800 cm\(^{-1}\), the second region between 2000 and 2800 cm\(^{-1}\), and the last region between 2800 and 3700 cm\(^{-1}\) were detected. First of all, typical of metal oxide single bonds in the bending mode were obtained at around 447 cm\(^{-1}\), 540 cm\(^{-1}\), and 613 cm\(^{-1}\). Especially, Cr–O stretching vibrations at 540 cm\(^{-1}\) and 613 cm\(^{-1}\) prove the synthesized \( \text{Cr}_2\text{O}_3 \)-Ps. A small peak is seen around 447 cm\(^{-1}\) due to Cr–O bending peak in the \( \text{Cr}_2\text{O}_3 \)-Ps structure. In addition, a H-bond peak between 2900 and 3600 cm\(^{-1}\) is observed stem from the -OH groups on the \( \text{Cr}_2\text{O}_3 \) surface [32]. In addition, a small H-OH peak is seen around 1658 cm\(^{-1}\) due to the absorbed moisture structure.

The X-ray spectrum of the synthesized \( \text{Cr}_2\text{O}_3 \)-Ps is shown in Fig. 1b. In this spectrum, there are peaks confirming the \( \text{Cr}_2\text{O}_3 \)-Ps, especially in the 20–70 2-theta (2θ) range. (012), (104), (110), (113), (202), (024), (116), (122), (214), and (300) peaks are visible. These peaks prove the desired \( \text{Cr}_2\text{O}_3 \)-Ps when compared with the literature [33].

SEM images of the obtained \( \text{Cr}_2\text{O}_3 \)-Ps were given in Fig. 2 at different magnifications. According to these images, the \( \text{Cr}_2\text{O}_3 \)-Ps structure was quite homogeneous and clean. No residual starting material and foreign phase are visible. It has been proven that the desired homogeneous \( \text{Cr}_2\text{O}_3 \)-Ps was obtained.

The hydrodynamic diameter measurements (± standard error) for the \( \text{Cr}_2\text{O}_3 \) structures used in this study are shown in Fig. 3a. According to this figure, DLS size distribution for the \( \text{Cr}_2\text{O}_3 \)-Ps is about 222 ± 125 nm and this distribution has been observed in a very narrow range (Fig. 3).

The EDX spectrum of the \( \text{Cr}_2\text{O}_3 \)-Ps obtained is given in Fig. 3b. On this spectrum, only peaks belonging to Cr and O are clearly seen. It is seen that the peaks of Cr in the structure are at 0.52, 5.48, and 5.91 keV values and are in harmony with the literature [33]. The Kα peak belonging to O is seen clearly at 0.54 keV. Other peaks on the spectrum are Au and Pd peaks during surface coating.

Thermal properties, another basic feature of \( \text{Cr}_2\text{O}_3 \)-Ps, are given in Fig. 3c with DTA and TGA thermograms. When these curves are examined in general, a mass loss of only 1.9% is observed due to the degradation of the starting material. This result proves that the \( \text{Cr}_2\text{O}_3 \) synthesis takes place with approximately 98.1% yield. In DTA thermograms of \( \text{Cr}_2\text{O}_3 \)-Ps, there is no thermal change between 30 and 1000 °C. This situation confirms the purity of the metal oxide. These results prove that the desired homogeneous and pure \( \text{Cr}_2\text{O}_3 \)-Ps were obtained.

The Cytotoxicity of \( \text{Cr}_2\text{O}_3 \)-Ps on Biochemical Parameters and the Functions of Spermatozoa

The cytotoxic effects of \( \text{Cr}_2\text{O}_3 \)-Ps were observed on functions such as the velocities of spermatozoa. The spermatozoa velocities which are the VSL, VCL, and VAP values significantly \( (p < 0.05) \) decreased after 100 µ/L concentration of \( \text{Cr}_2\text{O}_3 \)-Ps with compared control group. Besides, the lowest values of these velocities were observed
at the concentration of 1000 µg/L. However, after Cr₂O₃-Ps exposure, it was determined that there was no significant difference (p > 0.05) between 500 and 1000 µg/L in the velocities of spermatozoa.

According to our results, the cytotoxic effects as the functional deformations of spermatozoa were also investigated on the movement style of spermatozoa. For example, although the value of LIN decreased in dependent at 100 µ/L concentration of Cr₂O₃-Ps, it increased at 500 or 1000 concentrations of Cr₂O₃-Ps. While there was significant (p < 0.05) difference between these concentrations, it was not determined statistical difference (p > 0.05) with compared control group. The value of ALH showed decreased with increasing concentrations of Cr₂O₃-Ps and its decreasing was significantly (p < 0.05) different after 100 µg/L concentration with compare control group. The value of BCF and MAD values were statistically insignificant (p > 0.05) against Cr₂O₃-Ps concentrations compared with control groups (Table 1, Fig. 4).
According to our results, there was significant \( p < 0.05 \) decrease in the levels of CAT and SOD with increasing concentrations of \( \text{Cr}_2\text{O}_3\)-Ps. However, the CAT activity decreased after 500 µg/L concentration of \( \text{Cr}_2\text{O}_3\)-Ps, while the levels of SOD activity decreased after 100 µg/L concentration of \( \text{Cr}_2\text{O}_3\)-Ps compared to control group. Meanwhile, although the 100 mg/L concentration of \( \text{Cr}_2\text{O}_3\)-Ps significantly increased the MDA level compared to the control group \( p < 0.05 \), insignificant difference was observed \( p > 0.05 \) at tGSH levels in all groups (Table 2, Fig. 4).

Additionally, it was calculated effective concentrations for spermatozoa velocities against exposure of \( \text{Cr}_2\text{O}_3\)-Ps. The effective concentration (EC50) is the concentration of toxicant at which the toxicant is half of the maximum effective. In this study, the EC50 against exposure of \( \text{Cr}_2\text{O}_3\)-Ps was determined in values of VSL and VCL, 76.67 (65.55 to 89.67) µg/L and 87.77 (74.42 to 103.5) µg/L (Fig. 4), respectively.

### The Genotoxicity of \( \text{Cr}_2\text{O}_3\)-Ps on DNA of Spermatozoa

The detection of DNA fragmentation/damage using Comet assay, especially in spermatozoa, is a greatly beneficial and accurate way of explaining environmental toxicology (Singh et al., 1988). Furthermore, DNA damage in spermatozoa is related not only to toxic effects at that time but also affecting next generation of aquatic organisms. Thus, DNA fragmentations in spermatozoa occur after exposure environmental pollutants cause a reduction in hatching success as well as abnormalities in embryo development [34]. The present study will provide new useful data as being the first article investigating the genotoxic effect of \( \text{Cr}_2\text{O}_3 \) particles on the spermatozoa of rainbow trout (\textit{Oncorhynchus mykiss}) in the literature.

According to DNA evaluation by Comet assay in Fig. 5, the undamaged spermatozoa cells in IS were used as (-)
Fig. 3 Hydrodynamic size distribution (a), EDX spectrum (b), TGA and DTA thermograms (c) of Cr$_2$O$_3$ –P$_5$
control; on the other hand, the spermatozoa treated with hydrogen peroxide (H$_2$O$_2$) also known as (+) control had extremely damaged cells compared to the spermatozoa with Cr$_2$O$_3$-Ps. In addition, the spermatozoa of rainbow trout (Oncorhynchus mykiss) exposed to the different concentrations of Cr$_2$O$_3$-Ps revealed significantly higher DNA damages in when compared to the negative control. DNA fragmentation was detected in all spermatozoa after acute exposure. Using Comet assay images from Fig. 5, it is clearly seen that damages in DNA increase with gradually

| Parameters | $N=6$ | Mean ± S.E | 95% confidence interval for mean |
|------------|-------|------------|----------------------------------|
| VSL (µm/s) | Control | 31.45 ± 1.17$^c$ | 28.446 - 34.458 |
|            | 10 µg/L | 28.73 ± 0.61$^c$ | 27.170 - 30.292 |
|            | 50 µg/L | 28.75 ± 0.69$^c$ | 26.991 - 30.515 |
|            | 100 µg/L | 16.57 ± 0.44$^b$ | 15.439 - 17.705 |
|            | 500 µg/L | 15.14 ± 0.81$^{ab}$ | 13.051 - 17.231 |
|            | 1000 µg/L | 12.02 ± 0.52$^{ab}$ | 10.697 - 13.343 |
| VCL (µm/s) | Control | 120.86 ± 4.11$^c$ | 110.299 - 131.428 |
|            | 10 µg/L | 106.41 ± 2.62$^c$ | 99.683 - 113.142 |
|            | 50 µg/L | 105.65 ± 2.26$^c$ | 99.849 - 111.449 |
|            | 100 µg/L | 71.28 ± 6.32$^b$ | 55.028 - 87.529 |
|            | 500 µg/L | 50.90 ± 2.05$^a$ | 45.634 - 56.160 |
|            | 1000 µg/L | 38.67 ± 2.22$^a$ | 32.967 - 44.381 |
| VAP (µm/s) | Control | 54.12 ± 7.02$^c$ | 36.067 - 72.168 |
|            | 10 µg/L | 48.56 ± 4.75$^b$ | 36.343 - 60.778 |
|            | 50 µg/L | 46.23 ± 5.20$^c$ | 32.878 - 59.585 |
|            | 100 µg/L | 32.10 ± 2.62$^{ab}$ | 25.355 - 38.838 |
|            | 500 µg/L | 30.74 ± 3.05$^{ab}$ | 22.896 - 38.581 |
|            | 1000 µg/L | 19.50 ± 1.73$^a$ | 15.047 - 23.948 |
| LIN (%)    | Control | 26.06 ± 0.69$^{ab}$ | 24.290 - 27.826 |
|            | 10 µg/L | 24.86 ± 1.66$^{ab}$ | 20.591 - 29.128 |
|            | 50 µg/L | 26.02 ± 1.87$^{ab}$ | 21.206 - 30.839 |
|            | 100 µg/L | 21.11 ± 2.06$^a$ | 15.819 - 26.402 |
|            | 500 µg/L | 29.97 ± 1.93$^b$ | 25.006 - 34.942 |
|            | 1000 µg/L | 19.50 ± 1.73$^a$ | 15.047 - 23.948 |
| BCF (Hz)   | Control | 5.46 ± 0.75 | 3.545 - 7.377 |
|            | 10 µg/L | 8.61 ± 1.43 | 4.938 - 12.279 |
|            | 50 µg/L | 5.04 ± 1.20 | 1.952 - 8.135 |
|            | 100 µg/L | 5.73 ± 1.21 | 2.614 - 8.853 |
|            | 500 µg/L | 6.52 ± 1.38 | 2.980 - 10.050 |
|            | 1000 µg/L | 5.63 ± 0.81 | 3.561 - 7.699 |
| ALH (µm)   | Control | 30.31 ± 3.85$^b$ | 20.417 - 40.198 |
|            | 10 µg/L | 21.61 ± 1.51$^{ab}$ | 17.730 - 25.485 |
|            | 50 µg/L | 22.42 ± 1.75$^{ab}$ | 19.715 - 26.923 |
|            | 100 µg/L | 19.45 ± 1.67$^a$ | 15.172 - 23.730 |
|            | 500 µg/L | 17.93 ± 1.16$^a$ | 14.953 - 20.902 |
|            | 1000 µg/L | 16.07 ± 1.24$^a$ | 12.876 - 19.270 |
| MAD (°)    | Control | 0.04 ± 0.006 | 0.021 - 0.054 |
|            | 10 µg/L | 0.03 ± 0.004 | 0.018 - 0.040 |
|            | 50 µg/L | 0.04 ± 0.008 | 0.018 - 0.060 |
|            | 100 µg/L | 0.03 ± 0.004 | 0.023 - 0.043 |
|            | 500 µg/L | 0.03 ± 0.004 | 0.020 - 0.041 |
|            | 1000 µg/L | 0.04 ± 0.005 | 0.022 - 0.048 |

Data are presented mean ± SE of values. Different letters as $^a,b,c$ show differences between groups ($p<0.05$)
Fig. 4 The kinematics, biochemical stress indices and EC50 values of spermatozoa after Cr₂O₃-Ps exposure. Data are presented as mean ± SE of values. Different letters as a, b, c show differences between groups (p < 0.05).
Table 2  Biochemical stress indices of rainbow trout spermatozoa after exposed Cr₂O₃-Ps

| Parameters         | N = 6 | Mean ± S.E | 95% confidence interval for mean |
|--------------------|-------|------------|---------------------------------|
|                    |       |            | Lower bound | Upper bound               |
| CAT (U/mg protein) |       |            |             |                           |
| Control            | 20.44 ± 1.63ᵇ | 16.248     | 24.634     |
| 10 µg/L            | 20.17 ± 1.74ᵇ | 15.703     | 24.634     |
| 50 µg/L            | 18.26 ± 0.97ᵃᵇ | 15.765     | 20.746     |
| 100 µg/L           | 17.94 ± 1.14ᵃᵇ | 15.009     | 20.865     |
| 500 µg/L           | 13.48 ± 1.57ᵃᵇ | 9.441      | 17.519     |
| 1000 µg/L          | 12.51 ± 1.13ᵃᵇ | 9.606      | 15.422     |
| SOD (U/mg protein) |       |            |             |                           |
| Control            | 6.99 ± 0.62ᵇ  | 5.393      | 8.583      |
| 10 µg/L            | 5.52 ± 0.18ᵃᵇ | 5.051      | 5.985      |
| 50 µg/L            | 5.81 ± 0.33ᵃᵇ | 4.957      | 6.654      |
| 100 µg/L           | 4.76 ± 0.25ᵃ  | 4.110      | 5.407      |
| 500 µg/L           | 4.43 ± 0.33ᵃᵇ | 3.575      | 5.275      |
| 1000 µg/L          | 4.30 ± 0.33ᵃᵇ | 3.445      | 5.162      |
| MDA (nmol/mg protein) |     |            |             |                           |
| Control            | 1.43 ± 0.04ᵃ  | 1.318      | 1.535      |
| 10 µg/L            | 1.58 ± 0.10ᵃᵇ | 1.328      | 1.835      |
| 50 µg/L            | 1.52 ± 0.04ᵃᵇ | 1.415      | 1.631      |
| 100 µg/L           | 1.83 ± 0.11ᵇ  | 1.554      | 2.102      |
| 500 µg/L           | 1.60 ± 0.09ᵃᵇ | 1.375      | 1.828      |
| 1000 µg/L          | 1.60 ± 0.12ᵃᵇ | 1.306      | 1.897      |
| TGSH (nmol/mg protein) |     |            |             |                           |
| Control            | 15.92 ± 1.14  | 12.994     | 18.846     |
| 10 µg/L            | 14.68 ± 0.98  | 12.157     | 17.207     |
| 50 µg/L            | 16.53 ± 1.88  | 11.694     | 21.369     |
| 100 µg/L           | 16.40 ± 1.11  | 13.541     | 19.264     |
| 500 µg/L           | 14.84 ± 1.31  | 11.463     | 18.218     |
| 1000 µg/L          | 18.07 ± 1.91  | 13.152     | 22.983     |

Data are presented mean ± SE of values. Different letters as ᵐᵃᵇᶜ show differences between groups (p < 0.05)

Fig. 5  The different types of DNA damages in rainbow trout spermatozoa against different concentrations of Cr₂O₃-Ps. Each comet represents DNA fragmentations except (−) control
augmented concentrations of Cr$_2$O$_3$ particles. Thus, effects of Cr$_2$O$_3$ particles on DNA fragmentation of spermatozoa have been detected and described as dose-dependent manner.

The DNA fragmentations in Comet assay on spermatozoa after exposure of Cr$_2$O$_3$ particles is shown in Fig. 6A. The comparison of tail length (pixels), which is length of the tail in pixels, values between the negative and positive controls as well as the different concentrations of Cr$_2$O$_3$-Ps is given in Fig. 6B as a boxplot. Once rainbow trout spermatozoa (Oncorhynchus mykiss) cells exposure with increased particle concentrations of Cr$_2$O$_3$, the DNA integrity (also known as nucleoid structure) was lost and fragmented DNA molecules have longer tail lengths.

Tail DNA (%) means the percentage of the DNA in the tail content of a comet DNA. Besides, head DNA (%) is the percentage of the DNA in the head content of a comet DNA. As shown in Fig. 7, head DNA (%) values have been ranging from 86.88 ± 7.76 to 75.79 ± 10.66% conversely to tail DNA (%) depending on the specific concentration of Cr$_2$O$_3$ particles. Significantly higher DNA damages have been

**Fig. 6** (A) The different representative images of DNA fragmentations using Comet assay. (B) The comparison of tail length (pixels) values between the negative and positive controls as well as the different concentrations of Cr$_2$O$_3$-Ps. The box-plot graph shows the distribution of tail length (pixels) values of spermatozoa, and the horizontal lines indicate the median values. Each experiment was done as duplicated with having 100 spermatozoa per condition analyzed. The lowercase letters above the box-plot are significantly different. Significant from the negative control (a); significant from the positive control (b); significant from (c); significant from (d); significant from (e); significant from (f); significant from (g). NS, not significant
observed with increasing concentrations of \( \text{Cr}_2\text{O}_3 \) particles from the data of tail DNA (\%) in Fig. 7. In contrast to this data, the spermatozoa exposed to \( \text{Cr}_2\text{O}_3 \) particles exhibited lower DNA damages compared to cells treated with \( \text{H}_2\text{O}_2 \), (+) control (Fig. 7).

The spermatozoa in the negative control had undamaged cells approximately 60\% according to DNA evaluation by Comet assay from Fig. 8. The spermatozoa exposed to 100, 500, and 1000 \( \mu\text{g/L} \) of \( \text{Cr}_2\text{O}_3 \) particles showed significantly higher DNA damages compared to the negative control. The different levels of DNA damages except class 0 were detected in all spermatozoa cells starting from the concentration of 50 \( \mu\text{g/L} \) \( \text{Cr}_2\text{O}_3 \) particles. Moreover, spermatozoa in class 0 were not observed for \( \text{Cr}_2\text{O}_3 \) particles having 1000 \( \mu\text{g/L} \) concentration.

Values related to tail intensity (pixels), head intensity (pixels), tail DNA (\%), head DNA (\%), tail length (pixels), and tail moment, which is defined as tail length (pixels) \( \times \) tail DNA (\%) in Table 3 and Table 4.

**Discussion**

Within the scope of the study, the toxicology and environmental effects of \( \text{Cr}_2\text{O}_3 \) particles, which are widely used industrially, were studied. It is promising in many materials...
such as optical storage systems, solar panels, photovoltaic cells, photocatalytic systems, and UV light emitters. It is also widely used in industry for H storage, wear-resistant materials, catalyst, dyes, pigments, and some digital recording systems [4, 5]. Nano-sized Cr$_2$O$_3$ particles (less than 100 nm) have negative effects on the ecosystem. These effects have been shown in many studies in the literature [13–16]. Environmental toxicity of nano-sized Cr$_2$O$_3$ structures seems to be higher than micro-scale structures due to the fact that they are easier to interfere with living systems. However, this study was designed to investigate the effects of Cr$_2$O$_3$ particles formed as a result of industrial pollution. In today’s industry, Cr$_2$O$_3$ particles are used in many applications. Cr$_2$O$_3$ is used extensively in the ceramic, cement, ink, and catalyst industries. It is also used as a pigment and refractory material. In these industries, the size of industrial Cr$_2$O$_3$ particles that threaten today’s ecosystem is usually above 100 nm. Thus, Cr$_2$O$_3$ particles around 200 nm (222 ± 125 nm) were used in the study. As a result, Cr$_2$O$_3$ sizes in industrial pollution show a wide range distribution.

### Table 3

The comparisons between the percentages of DNA in head (%), DNA in tail (%), and tail moment using Comet assay of rainbow trout spermatozoa. The different concentrations of Cr$_2$O$_3$-Ps, (-) and (+) controls expressed as median (min–max)

| Groups          | DNA in head (%) Median (min–max) | DNA in tail (%) Median (min–max) | Tail Moment Median (Min – Max) |
|-----------------|----------------------------------|----------------------------------|--------------------------------|
| (-) Control     | 82.99 (70.99–93.4) bcfg          | 17.01 (6.6–29.01) bcfg          | 0.3 (0.07 – 2.03) bcfg         |
| (+) Control (H$_2$O$_2$) | 79.57 (7.69–97.22) acdef         | 20.43 (0.28–92.31) acdef         | 0.55 (0.06 – 23.68) acdef       |
| 10 µg/L         | 88.79 (67.44–99.52) acdef         | 11.21 (0.48–32.56) acdef         | 0.23 (0 – 2.3) bcfg             |
| 50 µg/L         | 85.57 (67.53–97.29) acdef         | 14.43 (2.71–32.47) acdef         | 0.22 (0.03 – 2.98) bcfg         |
| 100 µg/L        | 83.71 (54.85–94.53) bcfg          | 16.29 (5.47–45.15) bcfg          | 0.4 (0.06 – 6.32) bcfg          |
| 500 µg/L        | 79.18 (57.79–91.63) ced           | 20.82 (8.37–42.21) ced           | 0.38 (0.09 – 8.02) ced          |
| 1000 µg/L       | 78.73 (37.82–87.13) ced           | 21.27 (12.87–62.18) ced          | 0.6 (0.14 – 6.84) ced           |

The values (n = 100) are median (min–max) with common superscripts in the same line are significantly different

Min, minimum; Max, maximum

aSignificant from the negative control (a)
bSignificant from the positive control (b)
cSignificant from (c)
dSignificant from (d)
eSignificant from (e)
fSignificant from (f)
gSignificant from (g)

### Table 4

The dose-dependent effects of Cr$_2$O$_3$-Ps on genotoxicity parameters of rainbow trout spermatozoa after acute exposure

| Parameters        | Head intensity (pixels) | DNA in head (%) | Tail intensity (pixels) | Tail length (pixels) | DNA in tail (%) | Tail moment |
|-------------------|------------------------|-----------------|------------------------|----------------------|-----------------|-------------|
| (-) Control       | 47,183.44 ± 10,921.84 bcfg | 83.72 ± 5.3 bcfg  | 8814.06 ± 2519.2       | 2.34 ± 1.51 by      | 16.28 ± 5.3 bcfg  | 0.42 ± 0.39 ft |
| (+) Control (H$_2$O$_2$) | 28,358.78 ± 18,114.05 acdef | 70.73 ± 21.89 acdef | 11,170.98 ± 14,060.22 df | 6.94 ± 8.6 acdef | 29.27 ± 22.06 acde | 3.12 ± 5.52 acdef |
| 10 µg/L           | 83,807.06 ± 55,965.65 acdef | 86.88 ± 7.76 abef | 10,941.36 ± 5579.83 bd | 2.64 ± 1.95 y      | 13.12 ± 7.76 y | 0.37 ± 0.49 bfg |
| 50 µg/L           | 44,825.24 ± 16,163.64 bfg | 84.71 ± 7.72 y | 8038.12 ± 5027.89 df | 2.68 ± 2.24 y      | 15.29 ± 7.72 df | 0.49 ± 0.62 bfg |
| 100 µg/L          | 47,042.28 ± 20,393.43 bfg | 81.76 ± 8.28 bx | 10,035.4 ± 6086.71     | 3.68 ± 3.35 y      | 18.24 ± 8.28 bx | 0.83 ± 1.25 bfg |
| 500 µg/L          | 38,465.52 ± 18,634.98 abed | 78.59 ± 8.82 bfg | 10,449.36 ± 8936.16    | 3.48 ± 3.96 y      | 21.41 ± 8.82 bfg | 0.84 ± 1.13 bed |
| 1000 µg/L         | 36,698.08 ± 14,178.36 abed | 75.79 ± 10.66 ycde | 11,496.38 ± 7660.65 cd | 4.04 ± 2.89 yf     | 24.21 ± 10.66 yf | 1.13 ± 1.24 caf |

The values (n = 100) are mean ± standard deviation with common superscripts in the same line are significantly different

aSignificant from the negative control (a)
bSignificant from the positive control (b)
cSignificant from (c)
dSignificant from (d)
eSignificant from (e)
fSignificant from (f)
gSignificant from (g)
from 100 nm. Therefore, a wide size distribution was preferred in this study.

This study investigated the cytotoxic effects of different concentrations (10, 50, 100, 500, and 1000 µL/L) of Cr2O3-Ps on spermatozoa of rainbow trout (Oncorhynchus mykiss) in vitro conditions. For this target, the cytotoxic effects were analyzed using DNA fragmentations in the velocities, movement styles, enzymatic activities, and DNA damages of spermatozoa; the end of the study was determined effective concentrations of Cr2O3-Ps.

In this study, Cr2O3-Ps caused changes in the physical and biochemical functions in spermatozoa of rainbow trout. In light of the literature for functional deformations, there are several results which are parallel with our data. For example, lower concentrations than 100 µg/mL concentration of Cr2O3-Ps showed minimum toxicity on viability or apoptosis of two human lung cell lines, BEAS-2B, and A549 [14]. Another hand, high concentrations such as 300 cm²/mL of Cr2O3-Ps had a negative on the function and mechanism of epithelial cells, monocyte/macrophage cells, human erythrocytes, and combined culture [15]. However, it was observed morphological damages and partially disintegration in body of Daphnia similis [18]. However, especially the functional negative effects were shown on the enzyme activities and DNA fragmentations of spermatozoa with dose-dependent manner of Cr2O3-Ps in our study. Similarly, it was determined the negative effects such as significant reduction in catalase and cell membrane damage and oxidative stress responses in ROS on Escherichia coli after exposure of Cr2O3-Ps [19]. The exposure to Cr2O3-Ps on green alga showed cytotoxic effects such as inhabitation growth, lowered chlorophyll content, and increasing ROS levels [17]. Other hand, it was found the negative biological functions such as longevity, reproduction, and growth parameters of Daphnia magna after Cr2O3-Ps exposure [13] and the diversity of bacterial communities and inhibited the enzyme activity analysis [16].

Nano or micro particles have also harmful effects on the functional structures of cells such as reduced cell viability and inhibited proliferation [35], reduction of spermatozoa velocities [36] with DNA damage and enzyme activities [35, 37–40]. In our results, the velocities (VSL, VCL, and VAP) and the movement styles (ALH and LIN) of spermatozoa were negatively affected by dose-dependent manner of Cr2O3-Ps, and these results were parallel with data of above literatures.

The cytotoxic effects of Cr2O3-Ps were observed changes in enzymatic activities in spermatozoa. Biochemical parameters of spermatozoa such as CAT, SOD, MDA, and tGSH as oxidative stress indices were measured after Cr2O3-Ps exposure. SOD catalyzes the conversion of superoxide (O₂⁻) radical to H₂O₂, while CAT carries out the conversion of H₂O₂ to water and oxygen. Therefore, the SOD-CAT system provides the first defense against oxygen toxicity [41]. According to our results, there was a significant decrease in the levels of CAT and SOD with increasing concentrations of Cr2O3-Ps. This decrease in both SOD and CAT levels may have been caused by the excessive ROS production induced by Cr2O3-Ps. The same results were reported by Afifi et al. [41]. They investigated the toxicity effect of Ag-NPs on Oreochromis niloticus and Tilapia zillii. While 2 mg/L Ag-NPs did not lead to any significant change in the SOD and CAT levels, concentration of 4 mg/L showed a significant reduction in the levels. Similarly, our previous study reported that SOD and CAT levels were significantly decreased after exposure to 100 mg/L of Fe₃O₄ NPs [42]. In our study, while MDA level significantly increased compared control group, tGSH levels did not change. MDA is an important biokarmer to determine lipid peroxidation. This increase in MDA level after exposure to the Cr2O3-Ps could be due to the depletion of the antioxidant system, which is consistent with the aforementioned results. Also, Cr ions produced from Cr2O3-Ps are incriminated from increased lipid peroxidation [43]. Additionally, our outcomes were enforced by the results of Adebayo et al. [44]. Other hand, it is well known that GSH protects the biological systems from oxidative stress. Mechanisms of change in the tGSH level may be different. For instance, excessive ROS production may have affected tGSH levels. Alternatively, the Cr ions may have shown inhibition on GSH-synthesizing enzymes. Maybe both are involved.

Investigating the effects of UV radiation and hydrogen peroxide (used as oxidative agents) on rainbow trout, Oncorhynchus mykiss, spermatozoa, Dietrich et al. (2005) found a decrease in sperm motility and DNA integration after a long duration of UV. From our research, H₂O₂ effect on a spermatozoa cell has been observed as (+) control from Fig. 5 and Fig. 6A. Dose-dependent reductions in sperm motility and fertilizing features were significantly changed after spermatozoa were exposure to H₂O₂. Exposed of rainbow trout spermatozoa to some elements such as mercury and cadmium occurs an increase in DNA damage measured via Comet assay with brutal results in terms of sperm motility and hatching rate [45]. Unlike oocytes, fish spermatozoa have been known not to have efficient evolutionary defense mechanisms like DNA repairing against environmental pollutants even though having extremely condensed genetic material, DNA [46]. Furthermore, after exposure to physical/chemical stresses, spermatozoa have exhibited extreme sensitivity to oxidative stress, which is responsible for DNA fragmentation, because of not having enough antioxidant defense mechanism and extreme content in unsaturated fatty acids [47–51].
Conclusion

According to literatures, it is clear that chromium and its components are highly toxic for living organisms in the “Introduction” section. However, it has known that the cytotoxicity results can be very variable because there are many reasons such as cell type, the physiological functions of cell, and physical structures of particles. This study clarified the lack of information on the effects of Cr$_2$O$_3$-Ps on the reproductive functions of fish, especially sperm quality.

Finally, we concluded with all these results that may be useful data to determine the safety of Cr$_2$O$_3$-Ps in aquatic environment, ecotoxicology, and aquaculture sector.

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Data Availability The datasets used and/or analyzed during the current research are available from the corresponding author on reasonable request.

Declarations

Ethics Approval Not applicable. The local ethics committee was notified and committee approval was not necessary.

Consent to Participate Not applicable.

Consent to Publication Not applicable.

Competing Interests The authors declare no competing interests.

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