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ASCORBIC ACID AMELIORATES GENOTOXIC EFFECTS OF COBALT NANOPARTICLES AND COBALT CHLORIDE IN *IN VIVO DROSOPHILA* ASSAYS

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ABSTRACT

Nanoparticles are used in a wide variety of fields due to their many advantages in different industries, but knowledge about the genotoxic potential of nanoparticles is rather limited. It is known that genetic changes such as mutation, recombination, and DNA damage that occur in organisms are related to many diseases. Therefore, it is very important to determine such effects of nanomaterials. Ascorbic acid (AA), one of the water-soluble antioxidant micronutrients, can be found in many vegetables and fruits and acts as a cancer preventive agent by protecting cells against oxidative damage. In this study, the genotoxic effects of cobalt nanoparticles (CoNP, 40nm) and cobalt chloride ($CoCl_2$) and the antigenotoxic effect of AA against them were examined by means of both the Somatic Mutation and Combination Test (SMART) and the Single Cell Gel Electrophoresis Test (COMET) on *Drosophila melanogaster*. The SMART method is a fast, reliable, and economical *in vivo* test method based on the loss of heterozygosity genetic changes (point mutation, deletion, non-separation, and recombination) occurring in the wing imaginal disc cells. The alkaline COMET assay is a sensitive and fast technique for the detection of single-strand DNA breaks. In this study, both preliminary and concurrent applications were performed in the SMART method. It is seen that the damage induced by $CoCl_2$ and CoNPs in the pretreated group is higher than in the simultaneous application. On the other hand, simultaneous applications were made in the COMET study, and it was observed that AA decreased the genotoxicity with statistical significance.

KEYWORDS:
Drosophila, cobalt nanoparticle, ascorbic acid, genotoxicity, SMART assay, COMET assay.

INTRODUCTION

Nanotechnology is a field of study in which researchers have been closely involved in recent years, and the unique properties and applications of nanomaterials are being explored [1]. Nanotechnological products are used increasingly in many fields, such as engineering, medicine, pharmacology, agriculture, and the environment [2]. Moreover, because of their superior physicochemical properties, nanoparticles find wide use in medicine, food, clothing, and industrial applications. The widespread use of these materials is causing concern about the potential risks to organisms [3, 4]. As a result of the production, use, and excretion of nanoparticles, 40% of these products are mixed with soil, air, and water [5]. In that context, the effects of nanomaterials on human and environmental health and ways of protecting against possible adverse effects are important research topics.

Cobalt is found in rocks, soil, surface and ground waters, plants, and animals. Cobalt is also released from the burning of oil and coal, from automotive exhaust, and from industrial processes such as metalworking [6]. Because cobalt nanoparticles have high magnetism and a large active area, they are often used in the fields of electricity and electronics, such as in high-intensity magnetic storage, magnetic toners, and magnetic inks, and protecting mobile phones from electromagnetic wave radiation [7].

It is thought that components of vegetables and fruits such as vitamins, minerals, and polyphenols minimize the risk of chronic illnesses. Plant secondary metabolites, such as polyphenols and flavonoids, are quite important in terms of protecting the organisms at the cellular level against external factors [8-10]. Ascorbic acid (AA), which is found in many plants, has anticlastogenic, antimutagenic, antitumor, and protective effects against pesticides [11-17]. In this context, the genotoxic potentials of ionic cobalt and nanocobalt in this work have been demonstrated by the *Drosophila* SMART and COMET assays. In addition, it has been shown that genetic damage is eliminated by a AA.

MATERIALS AND METHODS

Chemicals. $CoCl_2$ (Cas No:7646-79-9), CoNP (Cas No:7440-48-4, Particle size:25-30nm), AA (Cas No: 50-81-7), ethyl methanesulfonate (EMS) (CAS No: 62-50-0), propionic acid, gum arabic, ethidium bromide (EtBr), ethylenediaminetetraacetic acid (EDTA), phosphate-buffered saline (PBS), and n-phenylthiourea were obtained from Sigma Aldrich. Sodium chloride (NaCl), sodium hydroxide (NaOH), low-melting-point agarose (LMA), normal-melting-point agarose (NMA), and tris were obtained from AppliChem Orthophosphoric acid, chloral hydrate, and glycerol were obtained from Merck, and agar was obtained from Alfa Aesar. $CoCl_2$ and CoNP were dissolved in ethyl alcohol, EMS, and AA were dissolved in distilled water.

Strains. In the *Drosophila* SMART assay, two *Drosophila* strains, *flr*^{3}/*TM3,Bd*^{s} (female) and *mwh/mwh* (male), were used [18]. The wild-type strain, Oregon R$^+$, was used in the COMET method. All strains used in the study were cultured at 25±1 ºC and 60% humidity.

***Drosophila melanogaster* Somatic Mutation and Recombination Test (SMART).** The SMART assay is based on the loss of heterozygosity in the wing imaginal disc cells. As described by Graf et al. (1984), transheterozygous larvae were obtained from mating virgin *flr*^{3}*(*flare) females and *mwh* males [19]. In concurrent applications, the eggs were collected in a clean nutrient medium for eight hours from mating virgin *flr*^{3} females and *mwh* males. After 72±4 hours, 3-day-old larvae were gathered under tap water with the aid of a sieve and transferred to vials containing 4.5 g of *Drosophila* instant medium (Formula 4-24, Carolina Biological Supply Co., Burlington, NC, ABD) and 9 ml of test chemicals (CoNP, $CoCl_2$, CoNP + AA, and $CoCl_2$ + AA, EMS, ethanol, and distilled water). In the preliminary study, larvae collected at the beginning of the second larval stage were exposed to different doses of AA for 24 hours and then exposed to 10 mM CoNP and 10 mM $CoCl_2$ at the beginning of the third larval stage. Adult flies were kept in 70% ethanol at +4 ºC until the wing preparations were prepared. Wings were removed and mounted in Faure's solution on microscope slides. Prepared wing preparations were examined at 40X magnification with an optical microscope.

***Drosophila melanogaster* Single Cell Gel Electrophoresis (COMET).** Eggs collected from the *Drosophila* Oregon R$^+$ strain during 8 hours were collected with a sieve when they were three-day-old larvae. *Drosophila* instant medium was prepared in application tubes with 4.5 g in 9 ml of solutions prepared for different application groups (CoNP, $CoCl_2$, CoNP + AA, and $CoCl_2$ + AA, EMS, ethanol, and distilled water). When the larvae were 96±4 hours old, they were collected under tap water with the aid of a sieve and stored in a 5% sodium hypochlorite solution for 2 minutes. Collection of 40-60 larvae from each application group was done according to the Irving (2005) [20] method. The hemocytes isolated from the larvae with the aid of a forceps were put into 1.5 ml centrifuge tubes containing n-phenylthiourea and PBS. The cells were centrifuged at 1300 rpm (300 x g) for 10 minutes at +4 ºC. After centrifugation, the supernatant was discarded, and the pellet was re-suspended with cold PBS solution. According to the Singh et al. (1988) [21] COMET procedure, 100 µl of hemocytes and 80 µl of LMA were mixed rapidly. The solution was spread over the slides, which had been NMA-coated one day before, and closed with cover glasses. After the slides were left on a cold plate for 10 minutes, the cover glasses were gently separated, and 80 µl of LMA was spread on them and they again were left on a cold plate for 10 minutes. After that, the cover glasses were removed from the slides, and the slides were placed in light-free slide-containing dishes containing lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM tris, 1% Triton X-100, and 1% N-Lauroylsarcosine sodium salt solution [pH = 10]). To prevent DNA damage, the following steps were performed under dim light. In this case, slides were left in the refrigerator for at least 1 hour and placed for 30 minutes in a horizontal electrophoresis tank filled with electrophoresis solution. The DNA was unwound by submerging the cells into electrophoresis buffer (pH 13.2) for 20 minutes then electrophoresis was carried out for 30 min at 300 mA and 25V. After the electrophoresis process, the slides were neutralized with 0.4 M tris (pH = 7.5) for 5 minutes twice. Then 50 µL of ethidium bromide (EtBr, 60 µg/mL) was added on each slide. For each preparation, 50 cells were counted on the fluorescence microscope (Nikon Eclipse E200) at 40X magnification. Tail intensity, tail moment, and tail length were used as evaluation parameters. All measurements were made on a fluorescence microscope in automatic measurement mode with the COMET-IV (Version 4.11) program.

Characterization of Nanoparticles. The CoNPs used in this study were synthesized by Prof. Dr. Ertuğrul Arpaç (Akdeniz University). Characteristic evaluations and particle sizing of NPs were performed with a Tecnai G2 F30 transmission electron microscope (TEM). Measurement of the zeta potentials was performed with the Malvern Zetasizer Nano-ZS, and density measurements were made with the Anton Paar DMA 4500 4 (National Nanotechnology Research Center, Ankara-Bilkent University) specific gravity and concentration meter.

Statistical Analysis. The data obtained in the SMART experiment were evaluated by means of a computer program (MICROSTA) prepared for *Drosophila* wing somatic mutation and recombination tests. Original and alternative hypotheses were calculated by using the binomial conditional test. Kastenbaum and Bowman (1970) [22] charts were used when original and alternative hypotheses were adopted or rejected. Student's t-test was used to evaluate the differences between the control groups and the treatment groups in evaluating the COMET test. A P value of >0.05 was considered for all applications in the COMET experiment.

RESULTS AND DISCUSSION

In this study, the antigenotoxic effect of AA against $CoCl_2$ and CoNP, which have genotoxic effects, was demonstrated. In the SMART assay, a total of 1904 wing counts were performed, with 80 wings for each concentration, excluding the EMS with high genotoxic activity. In the COMET method, 700 hemocyte counts were performed in total, 50 for each dose. To characterization of the CoNP TEM are used for determination, size distribution and morphology. Fig. 1 indicate TEM figure and zeta potential. The obtained average size of CoNP is 50nm and zeta potential value is -11.8 mV.

The Co ions used in our work are a necessary trace element for vitamin B12 formation, but they have the potential to create oxidative damage by complexing with ligands [23]. In addition, high doses of cobalt cause genotoxic damage in mammalian cells. Studies with cell lines have shown that cobalt causes DNA chain breaks, DNA-protein complex formation, and triggering of recombination [24]. CoNP, another chemical used in the study, is a nanomaterial with a wide range of industrial applications [25]. Elemental cobalt nanoparticles are used in a wide variety of fields on magnetic screening, magnetic toner, medical sensors, energy storage, and contrast enhancer effects [7, 26]. Antioxidants are molecules that can neutralize radicals or destroy oxidized molecules [10]. AA is a natural antioxidant compound found in fruits and vegetables [27].

Nanomaterials have a toxic potential as a result of their physicochemical properties, such as morphology, oxidant creations, surface function, and dissolution rate [28]. In addition, due to their small size, they easily pass through cell membranes and other biological barriers, causing cellular dysfunctions [29]. In 2015, it was determined that the worldwide market for nanomaterials is $1 trillion, and that amount reveals a serious nanomaterial exposure [2]. Nanoparticles make chemical pollutants in the environment less harmful and are used in the removal of groundwater [30], removal of heavy metals from wastewater [31], and cleaning of polluted air [32]. In recent years, magnetic metal nanoparticles such as cobalt and nickel have been used for magnetic resonance imaging [33]. The medical, occupational, and environmental exposure of nanoparticles used in wide-ranging and varied forms is causing concern [3]. The release of Co from the CoNP exposure leads to accumulation in the cells, which, in turn, causes cell viability to deteriorate [34]. One reason for the toxicity of cobalt-containing nanoparticles is thought to be the direct entry of cobalt into the cells and the dissolution of nanoparticles followed by an increase in cations in the growth medium [35].

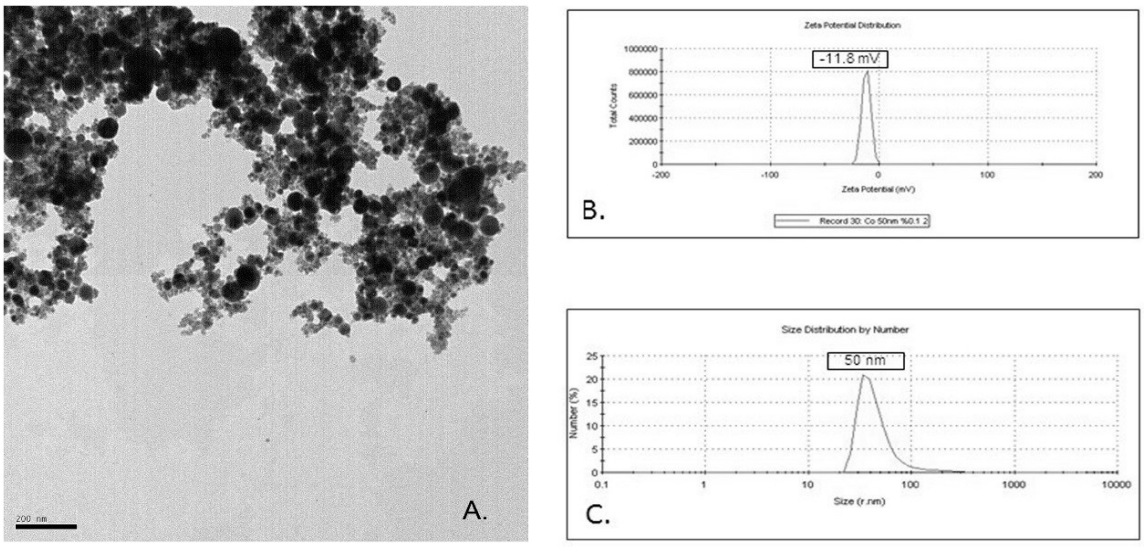


FIGURE 1
Characterization of cobalt nanoparticle (Co NP - 50 nm). Transmission Electron Microscopy (TEM) image of Co NP with Tecnai G2 F30 (A). Particle Size Distribution (B) and Zeta Potential Measurements(C)

D. melanogaster has been used by researchers in studies evaluating the toxicological potential of nanoparticles in reproductive, developmental, and genotoxicity tests [36-38]. The data obtained in our study of transheterozygous larvae are shown in Table 1 and 2. All doses of $CoCl_2$ and CoNP (0.1, 1, and 10 mM) showed statistically positive results when compared to ethyl alcohol, which was their solvent. Vales et al. (2013) [4] investigated the genotoxic effects of CoNPs by the *Drosophila* SMART assay, and they found increased dose-dependent genotoxicity. Nanomaterials have toxic potential, but there is no genotoxic effect of each nanoparticle. Alaraby et al. (2015) [39] in their studies investigating the effects of ZnO nanoparticles, observed that ZnO nanoparticles did not produce a genotoxic effect in the *Drosophila* SMART method but that they increased the expression of Hsp70 and p53 genes. Avalos et al. (2015) [40] investigated the toxicity of Ag nanoparticles of 4.7 nm size by the *Drosophila* SMART and found a statistically insignificant low toxicity.

Gornati et al. (2015) [33] found that CoNP caused cytotoxicity and increased Hsp70 levels in human ovarian cancer and human glioblastoma cell lines. DNA damage is a key mechanism in the toxicity of nanoparticles. The increase in the number of reactive oxygen species is caused by oxidative stress, which in this case causes DNA damage [41]. The COMET method is a sensitive and reliable method used by researchers to detect oxidative damage induced by nanoparticles and subsequent DNA damage [42-44]. The studies of Abudayyak et al. (2017) [45] on the cytotoxic and genotoxic effects of CoNPs on kidney cells did not reveal significant genotoxic effects in the COMET, while cell death was statistically significant. Liu et al. (2016) [46] investigated the cytotoxic and genotoxic effects of Co ions and CoNPs on the BRL-3A cell line. In that study, CoNP showed higher cytotoxicity than Co ions but also caused DNA damage in the COMET test. Ponti et al. (2009) [47] on Balb/3T4 cells and Colognato et al. (2008) [48] on human blood lymphocyte cells observed the genotoxic effects of CoNPs with COMET and micronucleus (MN) tests. Both studies have shown that CoNP has a genotoxic effect. *D. melanogaster* is a model organism used in human diseases, toxicological studies, and genetic and developmental studies [49]. *Drosophila* is also a model organism of interest in the investigation of nanomaterial-induced toxicity [38]. In our study, we found statistically significant single-stranded DNA damage in terms of all parameters for all concentrations of CoNP as compared to the solvent, ethyl alcohol and distilled water, according to the results obtained from the COMET test. Additionally, statistically significant single-stranded DNA damage was observed in terms of the tail moment and tail length parameters of all $CoCl_2$ doses compared to the ethyl alcohol, which is the solvent. EMS, which is a positive control group used in the study, was observed to cause genotoxic effects in terms of all parameters.

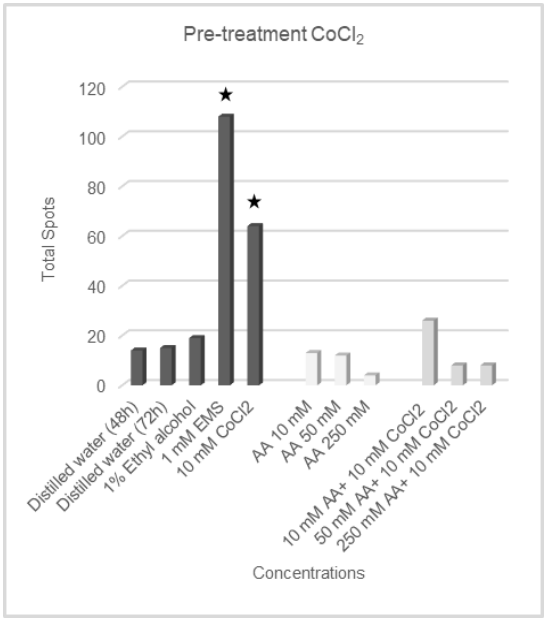


FIGURE 2
Pre-treatment of Ascorbic Acid Against $CoCl_2$ in SMART Assay

AA, also known as vitamin C, is a water-soluble vitamin and contributes to cellular protection mechanisms [50, 51]. AA is an antioxidant that protects the organism from oxidative stress by scavenging reactive oxygen species [52]. Bonilla et al. (2006) [53] investigated the protective effect of AA against paraquat toxicity and increased life span by increasing doses of AA. It is thought that AA inhibits cancer by inhibiting the formation of N-nitroso compounds and stimulating the immune system [54]. AA showed an anticlastogenic effect in the MN test with cyclophosphamide (CP), mitomycin-C (MMC) and bleomycin (BLM) hydrochloride in bone marrow cells [12]. AA has a protective role against chromosomal damage induced by doxorubicin (DXR) in human lymphocyte cells [13]. Yan et al. (2012) [55] observed a significant reduction in the level of 8-OHdG produced by ethanol in human lymphocyte cells pretreated with AA. In addition, a scavenging effect of hydroxyl radicals by AA was also observed in this study. Chang et al. (2012) [56] investigated brain development in mouse embryos and in newborn rats and observed the protective effect of AA on toxicity caused by lead. Olvera et al. (1995) [57] observed the protective effect of AA against gamma ray and CrO_3 toxicity in their studies using the *Drosophila* SMART method.

In the SMART assay, 48-hour-old larvae were exposed to different doses of AA for 24 hours in pre-treatment, and the same larvae were exposed to

a 10 mM dose of CoNP and $CoCl_2$ at the 72nd hour. In preliminary treatments, AA reduced the damage induced by $CoCl_2$ and CoNP with statistical significance (Fig.2 and Fig.3). According to the results obtained, all doses of AA had antigenotoxic effects in terms of all parameters (small single spot, large single spot, total mwh, and total clone) (Table 1).

Co-treatments were performed with 72±4-hour-old larvae that were exposed to CoNP, $CoCl_2$, and AA. We used 1 mM EMS as the positive control and distilled water and ethyl alcohol as the negative control. Similar to pre-treatments, AA reduced the genotoxicity in co-treatments (Fig.4 and Fig.5). According to the results obtained, AA showed a protective effect against genotoxicity induced by $CoCl_2$ and CoNP in terms of all doses and all parameters studied in the co-treatments (Table 2).

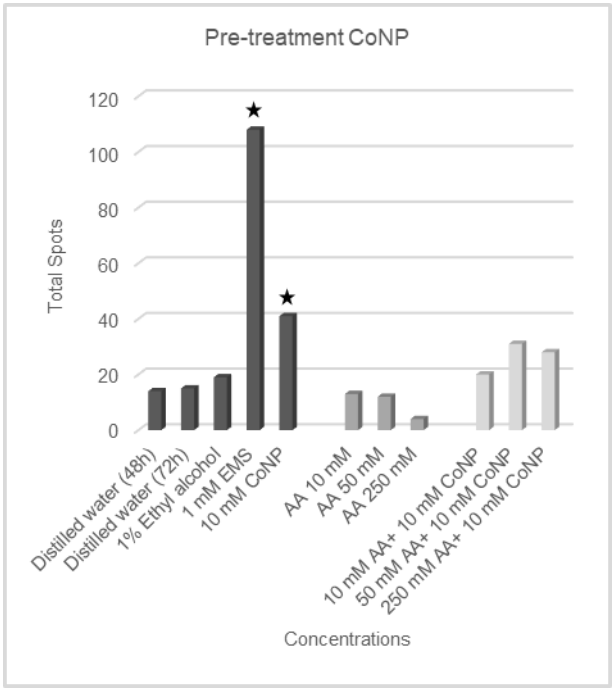


FIGURE 3
Pre-treatment of Ascorbic Acid Against CoNP in SMART Assay

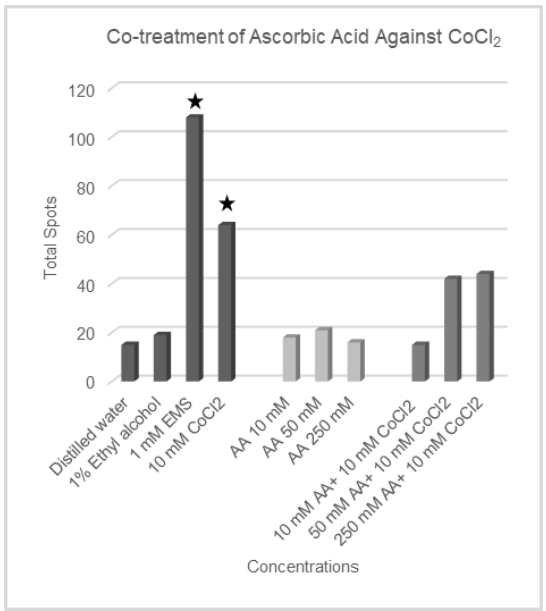


FIGURE 4
Co-treatment of Ascorbic Acid Against $CoCl_2$ in SMART Assay

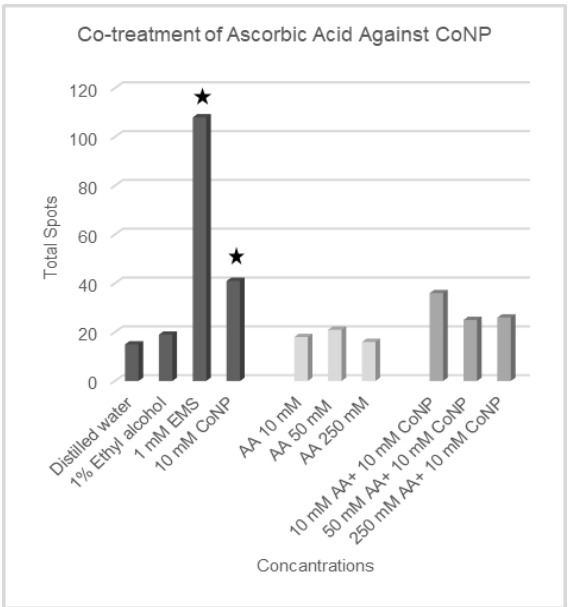


FIGURE 5
Co-treatment of Ascorbic Acid Against CoNP in SMART Assay

TABLE 1
In vivo* Antigenotoxic Effects of Pre-treatment of Ascorbic Acid Against $CoCl_2$ and CoNP Genotoxicity in *D. melanogaster

Compounds, concentration (mM)	Number of wings (n)	Small single spots (1-2 cells) (*m*=2)			Large single spot (>2cells) (*m*=5)			Twin spots (m=5)			Total *mwh* spots (m=2)			Total spots (m=2)			Frequency of clone formation (10^5 cells)
		No.	Fr.	D.	No.	Fr.	D.	No.	Fr.	D.	No.	Fr.	D.	No.	Fr.	D.	
Distilled water (48h)	80	12	(0.15)		2	(0.02)		0	(0.00)		14	(0.18)		14	(0.18)		0.72
Distilled water (72h)	80	13	(0.16)		2	(0.02)		0	(0.00)		15	(0.19)		15	(0.19)		0.77
%1 Ethyl alcohol (72h)	80	14	(0.18)	i	2	(0.02)	i	3	(0.04)	i	19	(0.24)	i	19	(0.24)	i	0.97
1 mM EMS (72h)	32	71	(2.29)	+	27	(0.87)	+	9	(0.29)	+	105	(3.39)	+	108	(3.48)	+	13.45
10 mM $CoCl_2$	80	53	(0.66)	+	9	(0.11)	+	2	(0.02)	i	64	(0.80)	+	64	(0.80)	+	3.28
10 mM CoNP	80	36	(0.45)	+	5	(0.06)	i	0	(0.00)	i	40	(0.50)	+	41	(0.51)	+	2.05
48-Askorbic acid (mM)																	
10	80	11	(0.14)	−	2	(0.02)	i	0	(0.00)	i	13	(0.16)	−	13	(0.16)	−	0.66
50	80	11	(0.14)	−	1	(0.01)	i	0	(0.00)	i	12	(0.15)	−	12	(0.15)	−	0.61
250	80	2	(0.02)	−	1	(0.01)	i	1	(0.01)	i	4	(0.05)	−	4	(0.05)	−	0.20
48±4 h Askorbic acid (mM) + 72±4 h 10 mM $CoCl_2$																	
10	80	22	(0.28)	−	3	(0.38)	−	1	(0.01)	i	26	(0.32)	−	26	(0.32)	−	1.33
50	80	5	(0.06)	−	2	(0.02)	−	1	(0.01)	i	8	(0.10)	−	8	(0.10)	−	0.41
250	80	5	(0.06)	−	2	(0.02)	−	1	(0.01)	i	8	(0.10)	−	8	(0.10)	−	0.41
48±4 h Askorbic acid (mM) + 72±4 h 10 mM CoNP (50 nm)																	
10	80	18	(0.22)	−	1	(0.01)	−	1	(0.01)	i	20	(0.25)	−	20	(0.25)	−	1.02
50	80	26	(0.32)	−	2	(0.02)	−	3	(0.04)	i	31	(0.39)	−	31	(0.39)	−	1.59
250	80	25	(0.31)	−	2	(0.02)	−	1	(0.01)	i	28	(0.35)	−	28	(0.39)	−	1.43

Fr., frequency D., statistical diagnosis; +, positive −, negative i, inconclusive; m=multiplication; probability levels= 0.05.

TABLE 2
In vivo* Antigenotoxic Effects of Co-treatment of Ascorbic Acid Against $CoCl_2$ and CoNP Genotoxicity in *D. melanogaster

Compounds, concentration (mM)	Number of wings (n)	Small single spots (1-2 cells) (*m*=2)			Large single spot (>2cells) (*m*=5)			Twin spots (m=5)			Total mwh spots (m=2)			Total spots (m=2)			Frequency of clone formation (10^5 cells)
		No.	Fr.	D.	No.	Fr.	D.	No.	Fr.	D.	No.	Fr.	D.	No.	Fr.	D.	
Distileted water (72h)	80	13	(0.16)		2	(0.02)		0	(0.00)		15	(0.19)		15	(0.19)		0.77
%1 Ethyl alcohol (72h)	80	14	(0.18)	i	2	(0.02)	i	3	(0.04)	i	19	(0.24)	i	19	(0.24)	i	0.97
1 mM EMS (72h)	32	71	(2.29)	+	27	(0.87)	+	9	(0.29)	+	105	(3.39)	+	108	(3.48)	+	13.45
10 mM $CoCl_2$	80	53	(0.66)	+	9	(0.11)	+	2	(0.02)	i	64	(0.80)	+	64	(0.80)	+	3.28
10 mM CoNP	80	36	(0.45)	+	5	(0.06)	i	0	(0.00)	i	40	(0.50)	+	41	(0.51)	+	2.05
72-Askorbic acid (mM) 72±4																	
10	80	13	(0.16)	i	5	(0.06)	i	0	(0.00)	i	18	(0.22)	i	18	(0.22)	i	0.92
50	80	16	(0.20)	i	3	(0.04)	i	2	(0.02)	i	21	(0.26)	i	21	(0.26)	i	1.08
250	80	14	(0.18)	i	1	(0.01)	i	1	(0.01)	i	16	(0.20)	i	16	(0.20)	i	0.82
72±4 h Askorbic acid (mM) + 72±4 h 10 mM $CoCl_2$																	
10	80	13	(0.16)	−	1	(0.01)	−	1	(0.01)	i	15	(0.18)	−	15	(0.18)	−	0.77
50	80	30	(0.37)	−	8	(0.10)	−	4	(0.05)	i	42	(0.52)	−	42	(0.52)	−	2.15
250	80	30	(0.37)	−	10	(0.12)	−	4	(0.05)	i	44	(0.55)	−	44	(0.55)	−	2.25
72±4 h Askorbic acid (mM) + 72±4 h 10 mM CoNP (50 nm)																	
10	80	30	(0.37)	−	4	(0.05)	−	2	(0.02)	i	36	(0.45)	−	36	(0.45)	−	1.84
50	80	23	(0.28)	−	2	(0.02)	−	0	(0.00)	i	25	(0.31)	−	25	(0.31)	−	1.28
250	80	19	(0.24)	−	6	(0.07)	−	1	(0.01)	i	26	(0.32)	−	26	(0.32)	−	1.33

Fr., frequency D., statistical diagnosis; +, positive −, negative i, inconclusive; m=multiplication; probability levels= 0.05.

Abraham (1994) [58] investigated the antigenotoxic effect of coffee against the mutagenic and carcinogenic chemicals cyclosphosphamide, diethylnitrosamine, procarbazine, mitomycin C, and urethane. In the study conducted with the *Drosophila* SMART method, coffee showed an antigenotoxic effect on total spot, twin clones and small spot clones against cyclosphosphamide, diethylnitrosamine, mitomycin C, and urea. Fernandes et al. (2017) [59] investigated the protective effect of vitexin, which has antimicrobial, antioxidant, and anti-inflammatory effects against the genotoxicity

induced by benzo[a]pyrene with the *Drosophila* SMART assay. The results obtained showed that vitexin exhibited an antigenotoxic effect in small single spots and in total clones. In another study conducted with the *Drosophila* SMART method, the protective effects of AA, lycopene, resveratrol, and $FeSO_4$ against the genotoxicity induced by 4-nitroquinoline-1-oxide (4-NQO), which is a carcinogenic and mutagenic chemical, were investigated. Antioxidants did not show a protective effect in the results obtained in the study [60]. Similarly, in the study of Vlastos et al. (2015) [61], the protective effect of Chios mastic oil, known for its anticarcinogenic potential, was evaluated by the *Drosophila* SMART method. The essential oil used in the study did not show a statistically significant protective effect against mitomycin C. Scolastic et al. (2008) [62] investigated the protective effect of lycopene, which has an antioxidant potential against n-nitrosodiethylamine, on a COMET assay with an HEPG2 cell line, and they observed a statistically significant protective effect at all doses. In another COMET study, vitamin C (AA) showed a high protective effect against nicotine exposure in mice [63].

In our study, it was observed in the COMET assay that, at all doses, AA did not cause genotoxic damage as compared to the control group of distilled water in terms of all parameters (Table 3). $CoCl_2$ and CoNP showed statistically positive results when compared with ethyl alcohol, which is a solvent. Antigenotoxicity evaluation showed a statistically significant decrease in tail length and tail moment of 10 mM $CoCl_2$ + 10 mM AA. Suspensions of 10 mM $CoCl_2$ + 50 mM AA and 10 mM $CoCl_2$ + 250 mM AA showed a statistically significant decrease in all parameters (Fig.6). The observed results obtained by co-administration of 10 mM CoNP with AA (10, 50, and 250 mM) were statistically significant reductions in all parameters (Fig.7).

The most important mechanism underlying the toxicity of nanomaterials is the production of reactive oxygen species (ROS). Excessive ROS production is caused by oxidative stress resulting in the degradation of normal physiological processes [29]. It has been determined that CoNPs enhance ROS production in *in vivo* and *in vitro* studies [28, 64-65]. Antioxidant molecules such as glutathione and cysteine act as redox buffers in different cell compartments and protect against reactive species. For that reason, antioxidants are used as protective agents against nanoparticle toxicity.

Antioxidants taken with the diet have a critical importance for reducing oxidative damage and reducing many chronic diseases. Antioxidant intake is thought to be one of the strategies for reducing nanomaterial-induced toxicity [29]. Nyga et al. (2015) [66] investigated the protective effect of AA (100μM) against the cytotoxicity induced by CoNP (5, 10, and 20 μg/ml) in alveolar macrophages. AA showed an effective protective effect at doses of 5 and 10 μg/ml of CoNP, while statistically different cell viability was not observed at 20 μg/ml AA. However, there are also studies showing that AA induces genotoxicity. In another study using the SMART method, AA showed a genotoxicity increase at a dose of 100 mM while there was reduced genotoxicity at 50 mM against doxorubicin [67].

TABLE 3
Protective effect of ascorbic acid against $CoCl_2$ and CoNP in COMET

Concentartion (mM)	Tail intense (%)	Tail moment (μm)	Tail length(μm)
Distilled water	0.85±0.29	0.10±0.03	21.44±0.80
Ethyl alcohol (%1)	1.52±0.23	0.18±0.39	21.88±1.01
EMS 5 mM	6.12±1.44***	1.91±0.36***	29.30±1.01***
10 mM $CoCl_2$	1.72±0.39$^{(\#)}$	0.58±0.09$^{***(\#)}$	26.10±0.95$^{***(\#)}$
10 mM CoNP	8.10±1.79$^{***(\#)}$	1.67±0.30$^{***(\#)}$	29.66±1.54$^{***(\#)}$
Ascorbic acid			
10	0.75±0.25	0.07±0.02	20.22±2.42
50	0.62±0.22	0.09±0.03	16.15±3.14
250	0.60±0.26	0.007±0.003	14.18±1.22
Ascorbic acid + 10 mM $CoCl_2$			
10	1.22±0.32$^{(\#)}$	0.17±0.29$^{(\#)}$	22.11±2.24$^{(\#)}$
50	1.04±0.27$^{(\#)}$	0.14±0.22$^{(\#)}$	20.18±1.82$^{(\#)}$
250	0.92±0.28$^{(\#)}$	0.11±0.25$^{(\#)}$	16.14±0.94$^{(\#)}$
Ascorbic acid + 10 mM CoNP			
10	0.96±0.34$^{(\#)}$	0.16±0.22$^{(\#)}$	21.21±1.13$^{(\#)}$
50	0.87±0.44$^{(\#)}$	0.11±0.26$^{(\#)}$	18.14±0.97$^{(\#)}$
250	0.76±0.38$^{(\#)}$	0.12±0.18$^{(\#)}$	14.28±0.82$^{(\#)}$

*0.01 < P < 0.05 **0.001 < P < 0.01 ***P < 0.001
$CoCl_2$ and CoNP administration compared to ethanol (Student's t-test)
EMS, ethyl methanesulfonate

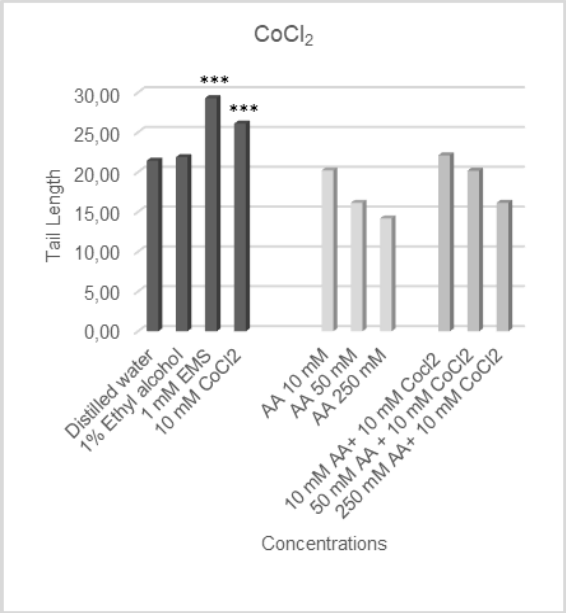


FIGURE 6
Antigenotoxic effect of AA against to $CoCl_2$ in COMET

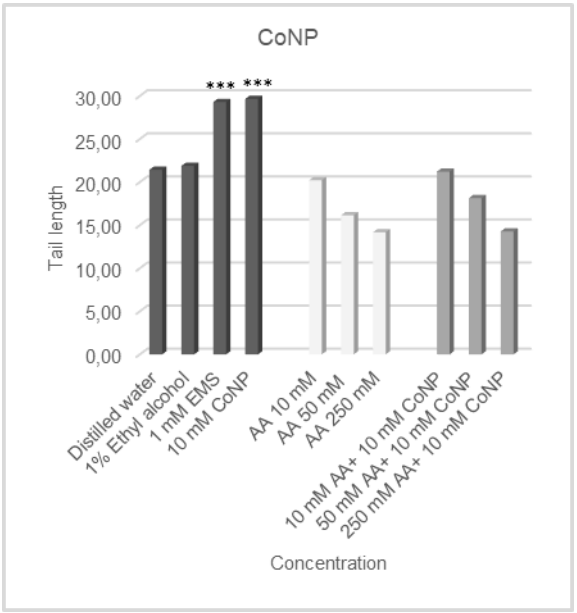


FIGURE 7
Antigenotoxic effect of AA against to CoNP in COMET

CONCLUSION

Drosophila SMART is a test system that allows for the identification of somatic mutations and abnormal recombinations associated with cancer in eukaryotic cells. *D. melanogaster* is also a model organism frequently used in the identification of DNA breaks. Those advantages make *Drosophila* an important model organism for investigating the genotoxicity induced by nanoparticles [68]. In this study, it was observed that CoNP caused a genotoxic effect when statistically compared to the control group. On the basis of the literature, this meaningful result is thought to be related to mechanisms such as an increase of ROS derivatives, inhibition of ATP synthesis, release of cytokines, up-regulation of heat-shock proteins, and induction of oxidative stress [69-74]. It is thought that the mechanism of the protective effect of AA against genetic damage induced by CoNP is mainly due to antioxidant effects. Comprehensive studies with different model organisms and test systems working with different mechanisms are needed to elucidate the mechanisms underlying the protective effect against induced genetic damage.

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