

## Research Article

# Studies on the Genotoxicity Behavior of Silver Nanoparticles in the Presence of Heavy Metal Cadmium Chloride in Mice

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Incredible rapid growth in the nanoparticles applications and development increases the daily human exposure to them but humans are exposed to many other pollutants in addition to nanoparticles that forced us to evaluate the effect of heavy metal cadmium chloride ( $\text{CdCl}_2$ ) coinjection on silver nanoparticles induced genotoxic risk in this study. Mice were injected into the abdominal cavity with single dose of Ag nanoparticles (20, 41, and 82 mg/kg) or  $\text{CdCl}_2$  (1.5 mg/kg) either separately or together simultaneously and sacrificed 24 hours later.  $\text{CdCl}_2$  cotreatment enhanced the induced dose-dependent sperm abnormality by Ag nanoparticles different doses as shown by the statistical significant decreases in both sperm concentration and motility and increases in the frequency of abnormal sperms and also potentiated the Ag nanoparticles induced chromosomal and DNA damage indicated by the statistical significant elevations in the frequencies of micronucleated polychromatic erythrocytes (MNPCEs) and DNA damage levels. Moreover, statistical elevations in malondialdehyde level and reductions in catalase activity were observed after  $\text{CdCl}_2$  coinjection with Ag nanoparticles compared with Ag nanoparticles treated groups' values. Ag nanoparticles induced sperm abnormality, clastogenicity, and genotoxicity were potentiated by heavy metal cadmium coinjection that threatens the human life and increases silver nanoparticles genotoxic risks.

## 1. Background

The last few years have seen an incredibly rapid growth in the use of nanomaterials such as metal nanomaterials in food, medicine, and industry, resulting in increasing human exposure to them. Silver (Ag) nanoparticles are one of the most commonly used metal nanoparticles in many consumer, medical, and industrial products such as water, toothpaste, shampoo, cosmetics, filters, kitchen utensils, toys, and food because of their characteristic antibacterial activity [1–4].

However, the extensive uses of Ag nanoparticles caused several negative health effects including hepatotoxicity, neurotoxicity, nephrotoxicity, and genotoxicity [5–7]. Genotoxicity and cytotoxicity of Ag nanoparticles have been evidenced in both *in vitro* and *in vivo* experimental systems. The inductions of chromosomal and DNA damage by Ag nanoparticles have been evidenced in several mammalian and fish cell [8–12]. Inductions of apoptosis and necrosis by

these nanoparticles were also shown in the cultured *HeLa* cells and intestinal epithelial cells [13, 14].

The *in vivo* induced genetic damage by Ag nanoparticles has been shown in different experimental systems. Ag nanoparticles have been shown to induce chromosomal aberrations and micronuclei in rat bone marrow cells [15]. Using comet assay DNA damage induction by Ag nanoparticles was indicated by the significant elevations in both single and double strand DNA breaks in rats and mice [16, 17]. High levels of  $\gamma$ -H2AX (a marker for double DNA strand breaks) also evidenced DNA damage inductions in zebra fish orally given Ag nanoparticles [18]. Moreover, polysaccharide coated Ag nanoparticles elevated the DNA damage markers (*p53* and *p38* proteins) in *Drosophila melanogaster* [19].

The embryo-toxicity of Ag nanoparticles also has been shown by the reported congenital malformations, reductions in mice fetus viability, apoptosis in mouse embryos at the blastocyst stage, reduction of implantation frequency, and

delay in postimplantation development of embryos [20–23]. Additionally, Ag nanoparticles have been shown to significantly reduce sperm counts and elevate the sperm abnormalities in mice and rats [24, 25].

In addition to these nanoparticles, there are many other substances to which humans are exposed directly or indirectly such as heavy metals. Cadmium (Cd) is one of the most important toxic environmental pollutants to which humans and animals are exposed because industrial and agricultural practices increase its level continuously in the environment [26]. Cd has been shown to induce micronuclei in polychromatic erythrocytes in both *tibia* bone marrow and peripheral blood in rats [27]. Also, the clastogenicity of Cd has been demonstrated by the development of chromosomal aberrations and sister chromatids exchanges [28, 29].

As previously shown the genotoxicity of either Ag nanoparticles or Cd was studied separately but the genotoxicity behavior of Ag nanoparticles combined with Cd was not studied until now in spite of human exposure to both Ag nanoparticles and Cd via contaminated air, drinking water, and even food. Therefore, the present study was designed to study the clastogenicity and genotoxicity of Ag nanoparticles in the presence of Cd in mice bone marrow, testes, and sperms. Micronucleus and comet assay were done to assess chromosomal and DNA damage, respectively. Sperm count, abnormality, and motility were also estimated to study the effect of Ag nanoparticles on sperm and thus on fertility.

## 2. Materials and Methods

**2.1. Animals.** Male Swiss Webster mice weighting 30–35 grams were obtained from the animal house of National Organization for Drug Control and Research (NODCAR). They were left in lab for one week under standard dark/light cycle to be acclimatized with the laboratory conditions and supplied with standard diet pellets and water that were given *ad libitum*. All experiments on animals were performed in accordance with The Institutional Animal Care and Use Committee (IACUC), Faculty of Science, Cairo University.

**2.2. Chemicals.** All chemicals were purchased from Sigma Aldrich Chemical Company. Cd was obtained in the form of white CdCl<sub>2</sub> powder and dissolved in deionized water to prepare the selected injected dose (1.5 mg/kg) that represents 25% of the computed 24-hour LD50 (5.98 mg/kg) in mice by the study of Ali [30]. While Ag nanoparticles were purchased in the form of grey nanopowder with size <100 nm, its purity was 99.5% and contains polyvinylpyrrolidone (PVP) as a dispersant. Ag nanoparticles were suspended in deionized distilled water to prepare the doses required to inject mice in both preliminary tests to detect their LD50 and the remaining experiments.

### 2.3. Characterization of Ag Nanoparticles

**2.3.1. X-Ray Diffraction (XRD).** Nano-Ag particles XRD patterns were measured using a charge coupled device diffractometer (XPRT-PRO, PANalytical, Netherlands). Using Scherrer's relationship ( $D = 0.9 k/B \cos h$ ) particle size

was calculated, where  $k$  is the wavelength of X-ray,  $B$  is the broadening of diffraction line measured as half of its maximum intensity in radians, and  $h$  is Bragg's diffraction angle. The particle size of sample has been estimated from the line width of XRD peak.

**2.3.2. Dynamics Laser Scattering (DLS).** Agglomeration size and zeta potential of Ag nanoparticles were detected by the routine work using Malvern Instrument Zetasizer Nano Series (Malvern Instruments, Westborough, MA) equipped with a He-Ne laser ( $\lambda = 633$  nm, max 5 mW).

**2.3.3. Transmission Electron Spectroscopy (TEM).** After sonication of Ag nanoparticles suspensions in Milli-Q water at 40 W for 25 min, drops of Ag suspensions were coated on carbon-coated copper TEM grids and dried, and finally TEM (a Tecnai G20, Super twin, double tilt) was operated at an accelerating voltage of 200 kV to Ag nanoparticles and to detect their morphology and particle average size.

**2.4. Determination of Ag LD50.** The lethal dose of Ag nanoparticles that causes the death of 50% of the animals (LD50) was determined by injecting mice intraperitoneally (i.p.) with each of the six dose levels of Ag nanoparticles 500, 1500, 2500, 3500, 4000, or 5000 mg/kg b.w., five mice per each group. Each group was observed for mortality, body weight effects, and the clinical signs of toxicity and the number of dead mice was monitored during the first 24 hours. After that, the LD50 was calculated using the computer software EPA probit analysis by aid of NCSS package software, version 10, and the three fractions (1/100, 1/50, and 1/25) of LD50 dose of Ag nanoparticles were tested in this study.

**2.5. Treatment Schedule.** Animals were divided randomly into eight groups of five animals each and injected i.p. First two groups were injected with distilled water (dist. H<sub>2</sub>O) (negative control group) or CdCl<sub>2</sub> at 1.5 mg/kg b.w. while the remaining six groups were injected with each of the three dose levels of Ag nanoparticles (20, 41, and 82 mg/kg b.w.) either alone (groups 3, 4, and 5) or simultaneously with CdCl<sub>2</sub> (groups 6, 7, and 8) and all groups were sacrificed 24 hours later.

**2.6. Sperm Abnormality.** Sperm abnormality was investigated by removing the cauda epididymides and cutting them into small pieces in Petri dish containing 2 mL saline. A small amount of sperm suspension was added to the cell counting hemocytometer plates and the total sperm number and motile sperm number were counted using a high-magnification microscope as described by Watanabe and Endo [31]. The sperm concentration and motility rate were calculated using the following equations:

$$\text{Sperm concentration} = \frac{\text{the total sperm number}}{(4 \times 10^4 \times 2)} \quad (1)$$

$$\text{Sperm motility rate} = \left( \frac{\text{the motile sperm number}}{\text{the total sperm number}} \right) \times 100.$$

Indeed, a small amount of sperm suspension was drawn and smeared on a slide, fixed for 10 min with methanol, stained for 1 hour with 1% eosin, and then washed with water. A total of 1000 sperms were counted to determine the proportion of malformed sperm using a high-magnification microscope:

$$\begin{aligned} &\text{The sperm malformation rate} \\ &= (\text{the malformed sperm number} \div 1000) \times 100. \end{aligned} \quad (2)$$

**2.7. Micronucleus Assay.** To detect the chromosomal damage, micronucleus assay was performed on bone marrow according to the method described by Schmid [32]: femur bone marrow cells are flushed down, spread on clean slide, air-dried, fixed, and finally stained for 5 min in May-Grunwald-Giemsa stain mixture and then mounted with Distrene 80, Dibutyl Phthalate Xylene (DPX). Two thousand polychromatic erythrocytes per animal were scored to determine the number of micronucleated polychromatic erythrocytes (MNPCEs). Also, polychromatic to normochromatic erythrocytes ratio (PCEs/NCEs) was determined per 1000 cells.

**2.8. Comet Assay.** The alkaline comet assay was done to detect both single and double strand breaks in sperms, testes, and bone marrow cells. Bone marrow cells and sperms obtained from epididymis were suspended into mincing solution, while small piece of testis, about 50 mg, was homogenized gently into mincing solution. According to Tice et al. [33] method, about 10,000 cells were mixed with 75  $\mu$ L of 0.5% low melting point agarose (Sigma) and spread on a fully frosted slide predipped in normal melting agarose (1%). After solidification, cells were lysed in cold lysis buffer (2.5 M NaCl, 100 mM EDTA, and 10 mM Tris, pH 10) with freshly added 10% DMSO and 1% Triton X-100 for 24 h at 4°C in dark. Subsequently, the slides were incubated in fresh alkaline buffer (300 mM NaOH and 1 mM EDTA, pH 13) for 20 min. The unwinding DNA was electrophoresed for 20 min at 300 mA and 25 V (0.90 V/cm) and neutralized in 0.4 M Trizma base (pH 7.5) and, finally, fixed in 100% cold ethanol, air-dried, and stored at room temperature until cells were scored. The extent of DNA migration for each sample was determined by simultaneous image capture and scoring of 100 cells stained with ethidium bromide at  $\times 400$  magnification using Komet 5 image analysis software developed by Kinetic Imaging, Ltd. (Liverpool, UK). The extent of DNA damage was evaluated using tail length, % tail DNA, and tail moment as DNA damage endpoints.

**2.9. Malondialdehyde Level and Catalase Activity Measurement.** In this study biochemical evaluation of malondialdehyde (MDA) level (marker of lipid peroxidation) and the antioxidant catalase (CAT) activity was done in bone marrow, testis, and epididymal sperms of all groups. According to the method described by Ohkawa et al. [34], the MDA level was determined by reacting the thiobarbituric acid (TBA) substance with MDA in acidic medium at temperature of 95°C for 30 min to form TBA reactive product and the absorbance of the resultant pink product was measured

spectrophotometrically at 534 nm. Results were expressed as nmol/g tissue used for bone marrow and testis and as nmol/ $10^6$  sperms for epididymal sperms.

On the other hand, CAT activity was measured using the method described by Aebi [35] that showed that CAT reacts with a known quantity of  $\text{H}_2\text{O}_2$ . The reaction is stopped after exactly 1 min with CAT inhibitor. In the presence of peroxidase, the remaining  $\text{H}_2\text{O}_2$  reacts with 3,5-dichloro-2-hydroxybenzene sulfonic acid and 4-aminophenazone to form a chromophore with color intensity inversely proportional to the amount of CAT in the original sample. Results were expressed as U/g tissue used for bone marrow and testis and as U/ $10^6$  sperms for epididymal sperms.

**2.10. Statistical Analysis.** Using the statistical software package SPSS 21 all data were analyzed at the significant level  $< 0.05$  by the independent sample *t*-test to test differences between each of the treated groups and the negative control group. One way analysis of variance (ANOVA) and regressions analysis curves were also used to test effect of Ag nanoparticles dose on the tested parameters.

### 3. Results

**3.1. Characteristic of Ag Nanoparticles.** Results of Ag nanoparticles characterization were summarized in Figure 1. The appearance of peaks at 44°, 64.4°, and 77° in XRD analysis curve confirmed the purchased form of Ag nanoparticles (Figure 1(a)) and by using Debye Scherrer's formula their nanocrystal size was confirmed and found to range from 20.8 to 277 nm. The zeta potential mean was 9.35 mV and the polydispersity index (PdI) as synonym to agglomeration size was 1 that indicated the high aggregation and agglomeration capacity of Ag nanoparticles in dist. deionized  $\text{H}_2\text{O}$  (Figures 1(b) and 1(c)). Indeed, TEM imaging of the ultrasonicated Ag nanoparticles showed their cubic structure increasing its surface area and its activity and confirmed the nanosize of Ag nanoparticles ( $56.67 \pm 9.77$  nm) in spite of presence of small agglomerates (Figure 1(d)).

**3.2. LD50 of Ag Nanoparticles.** Signs of mortality were observed on mice injected i.p. by the different doses of Ag nanoparticles (500, 1500, 2500, 3500, 4000, or 5000 mg/kg b.w.) including weakness, decreased motor activity, convulsions, abdominal swelling, and hind limbs paralysis. Using the probit analysis software, the 24 hours' lethality doses (from LD1 to LD99) of Ag nanoparticles were calculated and summarized in Table 1. As LD50 were found to be about 2056 mg/kg b.w., the tested three fractions of (1/100, 1/50, and 1/25) LD50 in this study were 20, 41, and 82 mg/kg b.w., respectively.

**3.3. Sperm Abnormality.** As shown in Table 2, groups treated with either  $\text{CdCl}_2$  (1.5 mg/kg) or Ag nanoparticles (20, 41, and 82 mg/kg) alone induced sperm abnormality as shown by the statistical significant increases ( $p < 0.001$ ) in frequency of abnormal sperms and decreases ( $p < 0.001$ ) in both sperm concentrations and sperm motility compared with the negative control group. On the other hand, simultaneous

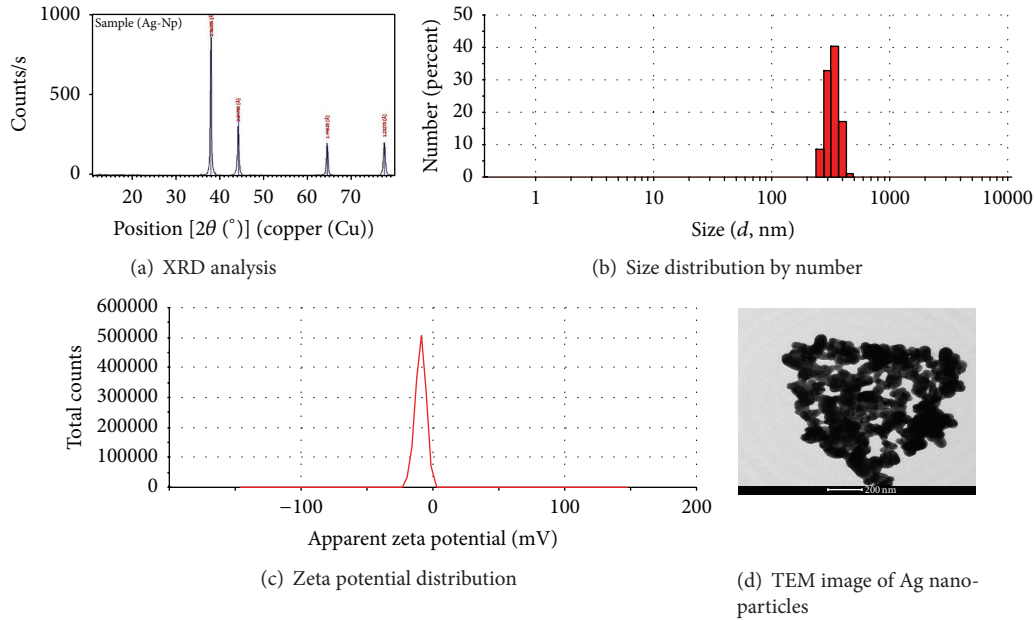


FIGURE 1: Characterization of Ag nanoparticles.

TABLE 1: The computed 24-hour lethality doses of Ag nanoparticles injected i.p. in male mice.

Percentile	Probit	Dose (mg/kg) Mean ± SE
1	2.6737	377.31 ± 266.47
5	3.3551	620.03 ± 335.51
10	3.7184	808.01 ± 368.37
20	4.1584	1113.45 ± 398.48
25	4.3255	1257.69 ± 406.39
30	4.4756	1403.09 ± 412.11
40	4.7467	1709.56 ± 422.15
50	5.0000	2056.26 ± 441.17
60	5.2533	2473.28 ± 491.44
70	5.5244	3013.51 ± 615.91
75	5.6745	3361.88 ± 730.21
80	5.8416	3797.40 ± 903.93
90	6.2816	5232.89 ± 1647.36
95	6.6449	6819.33 ± 2669.52
99	7.3263	11206.31 ± 6152.05

injection of CdCl<sub>2</sub> with Ag nanoparticles different doses enhanced the Ag nanoparticles induced sperm abnormality by the statistical significant increases in frequency in abnormal sperm and decreases in sperm concentrations and sperm motility (groups 6, 7, and 8) compared with those in groups treated with Ag nanoparticles alone (groups 3, 4, and 5). Representative photo for the observed abnormal sperms was shown in Figure 2.

One way ANOVA analysis showed that the dose of Ag nanoparticles caused statistical significant changes ( $p < 0.001$ ) in

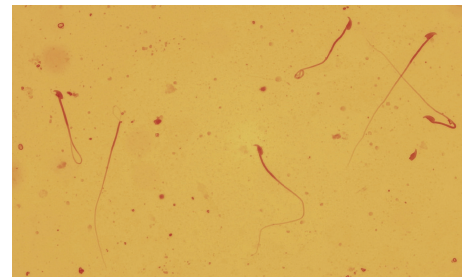


FIGURE 2: Representative photo for the observed abnormal sperms.

sperm concentration, sperm motility, and abnormal sperms frequency. Moreover, regression analysis curves indicated strong negative and positive correlations between both sperm concentration and motility and abnormal sperm frequency, respectively, and the different doses of Ag nanoparticles in both groups received Ag nanoparticles either alone or with Cd (Table 2, Figure 3).

**3.4. Results of Micronucleus Assay.** Chromosomal damage induction was estimated in this study by the frequency of micronucleated polychromatic erythrocytes (MNPCEs/1000 PCEs). Statistical significant elevations in the MNPCEs frequencies ( $p < 0.001$ ) were induced in groups treated with either CdCl<sub>2</sub> (1.5 mg/kg) or Ag nanoparticles (20, 41, and 82 mg/kg) compared with the negative control (Table 3). Moreover, CdCl<sub>2</sub> cotreatment with the different doses of Ag nanoparticles resulted in statistical significant elevations in the MNPCEs frequencies compared with Ag nanoparticles treated groups (Table 3).

Also, administration of either Cd or Ag nanoparticles alone resulted in statistical significant decreases ( $p < 0.001$ ) in the percentage of polychromatic erythrocytes (% PCEs)



TABLE 2: Sperm concentrations, sperm motility, and abnormal sperms frequency in mice injected i.p. with Ag nanoparticles or/and CaCl<sub>2</sub>.

Group	Treatment (dose mg/kg)	Sperm concentration (10 <sup>6</sup> /mL)	Sperm motility (%)	Abnormal sperms (%)
1	Negative control	1.44 ± 0.04	77.04 ± 1.68	5.20 ± 1.30
2	Cd <sub>(1.6)</sub>	0.58 ± 0.15 <sup>a</sup>	29.63 ± 3.26 <sup>a</sup>	58.20 ± 4.66 <sup>a</sup>
3	Ag <sub>(20)</sub>	1.18 ± 0.06 <sup>a</sup>	55.09 ± 3.55 <sup>a</sup>	18.40 ± 3.21 <sup>a</sup>
4	Ag <sub>(41)</sub>	0.92 ± 0.07 <sup>a</sup>	30.44 ± 2.48 <sup>a</sup>	37.6 ± 2.30 <sup>a</sup>
5	Ag <sub>(82)</sub>	0.45 ± 0.05 <sup>a</sup>	21.92 ± 2.05 <sup>a</sup>	59.00 ± 2.24 <sup>a</sup>
6	Ag <sub>(20)</sub> + Cd	0.98 ± 0.05 <sup>a,b***</sup>	35.47 ± 2.63 <sup>a,b***</sup>	31.20 ± 2.38 <sup>a,b***</sup>
7	Ag <sub>(41)</sub> + Cd	0.70 ± 0.08 <sup>a,b***</sup>	23.86 ± 2.96 <sup>a,b***</sup>	52.20 ± 2.59 <sup>a,b***</sup>
8	Ag <sub>(82)</sub> + Cd	0.36 ± 0.04 <sup>a,b***</sup>	10.71 ± 1.72 <sup>a,b***</sup>	74.60 ± 4.56 <sup>a,b***</sup>
Groups 3, 4, and 5		$F = 194, p < 0.001$	$F = 193, p < 0.001$	$F = 300, p < 0.001$
Groups 6, 7, and 8		$F = 137, p < 0.001$	$F = 124, p < 0.001$	$F = 216, p < 0.001$

Results were expressed as mean ± SD. <sup>a</sup>Statistically significantly different from the negative control group at  $p < 0.001$  and <sup>b</sup>statistically significantly different from the comparable nano-Ag treated group at  $**p < 0.01$  and  $***p < 0.001$ , respectively, using Student's *t*-test. One way ANOVA was used to test the effect of different doses of Ag nanoparticles on the tested parameters.

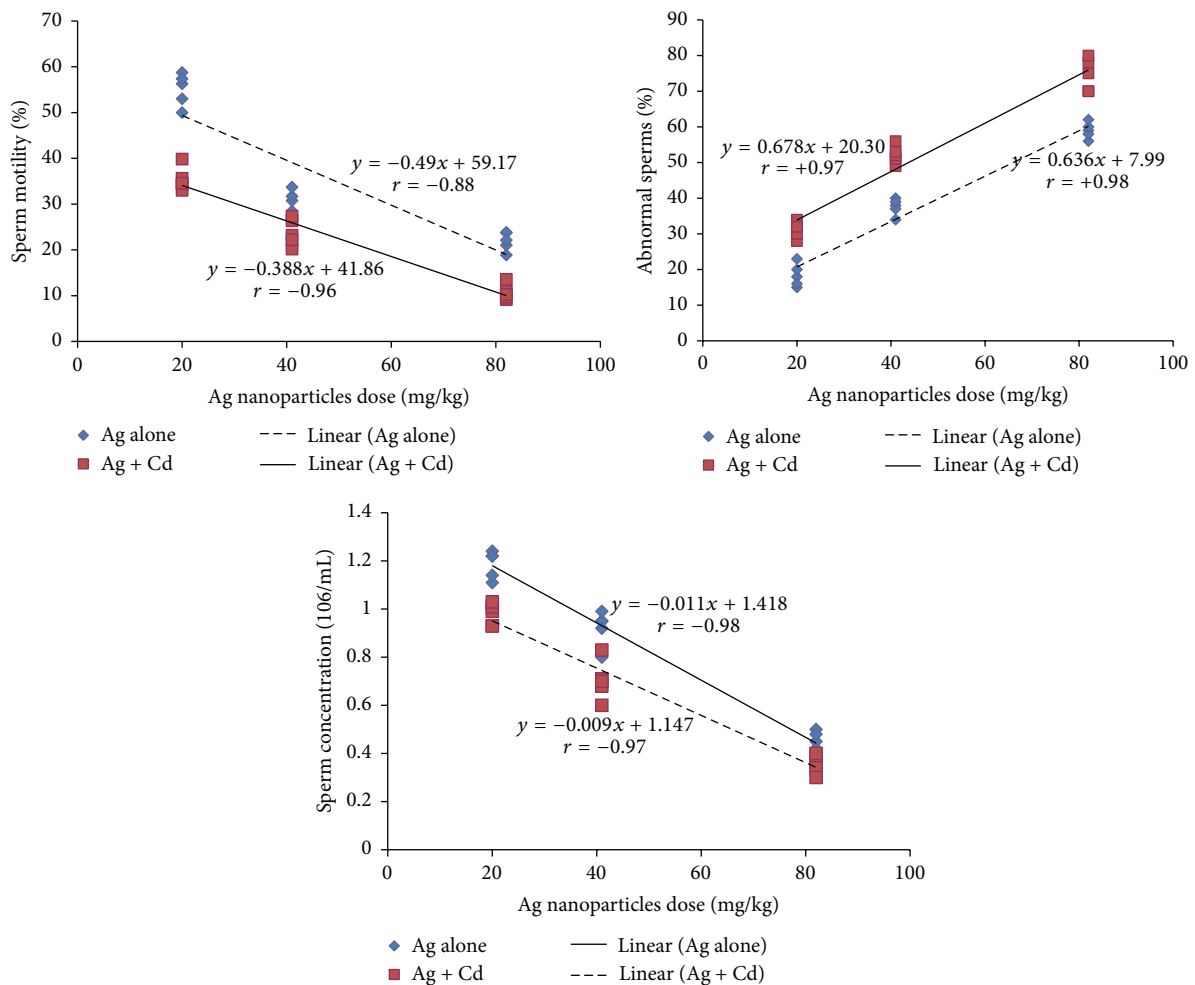


FIGURE 3: Regression lines and correlation coefficients between sperm concentrations, sperm motility, or frequency of abnormal sperms and the different doses of Ag nanoparticles in mice injected i.p. with Ag nanoparticles either alone or with CaCl<sub>2</sub>.

TABLE 3: The MNPCEs frequencies and PCEs percentage in mice injected i.p. with Ag nanoparticles or/and CaCl<sub>2</sub>.

Group	Treatment (dose mg/kg)	MNPCEs/1000 cells	% PCEs
1	Negative control	5.20 ± 2.17	49.80 ± 1.48
2	Cd <sub>(1.6)</sub>	47.60 ± 3.36 <sup>a</sup>	35.80 ± 2.39 <sup>a</sup>
3	Ag <sub>(20)</sub>	47.20 ± 2.17 <sup>a</sup>	41.80 ± 1.30 <sup>a</sup>
4	Ag <sub>(41)</sub>	73.60 ± 6.02 <sup>a</sup>	30.60 ± 2.41 <sup>a</sup>
5	Ag <sub>(82)</sub>	94.40 ± 5.94 <sup>a</sup>	22.20 ± 3.81 <sup>a</sup>
6	Ag <sub>(20)</sub> + Cd	73.60 ± 6.65 <sup>a,b***</sup>	36.20 ± 2.77 <sup>a,b**</sup>
7	Ag <sub>(41)</sub> + Cd	104.60 ± 5.73 <sup>a,b**</sup>	25.80 ± 1.92 <sup>a,b***</sup>
8	Ag <sub>(82)</sub> + Cd	144.60 ± 5.73 <sup>a,b**</sup>	17.40 ± 1.14 <sup>a,b***</sup>
Groups 3, 4, and 5		$F = 105, p < 0.001$	$F = 173, p < 0.001$
Groups 6, 7, and 8		$F = 149, p < 0.001$	$F = 110, p < 0.001$

Results were expressed as mean ± SD. <sup>a</sup>Statistically significantly different from the negative control group at  $p < 0.001$  and <sup>b</sup>statistically significantly different from the comparable nano-Ag treated group at  $**p < 0.01$  and  $***p < 0.001$ , respectively, using Student's *t*-test. One way ANOVA was used to test the effect of different doses of Ag nanoparticles on the tested parameters.

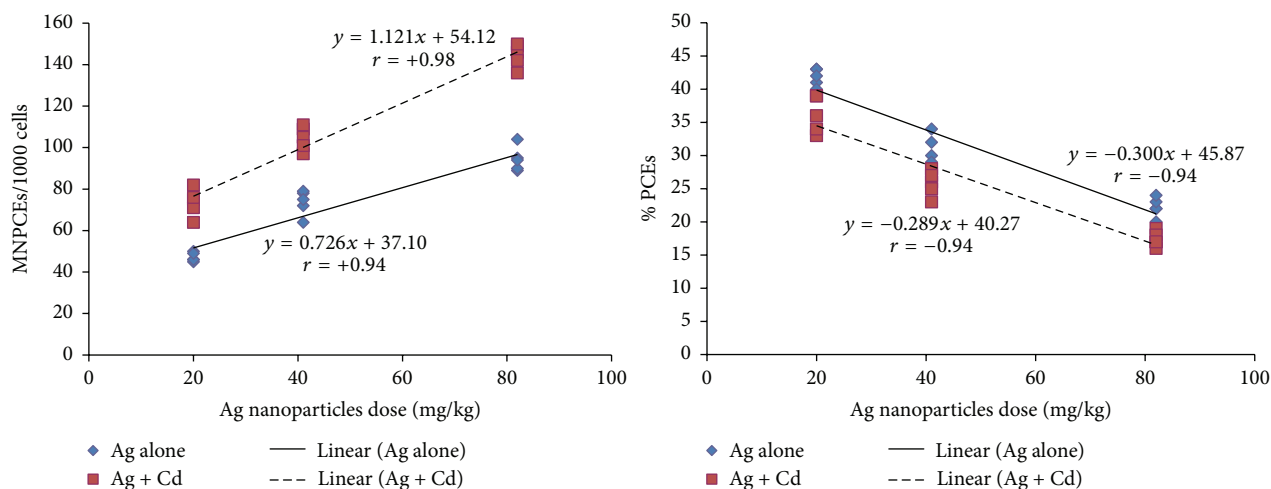


FIGURE 4: Regression lines and correlation coefficients between frequency of MNPCEs or percentage of PCEs and the different doses of Ag nanoparticles in mice injected i.p. with Ag nanoparticles either alone or with CaCl<sub>2</sub>.

compared with the negative control level and the cotreatment of CdCl<sub>2</sub> with Ag nanoparticles different doses statistically decreased ( $p < 0.001$ ) % PCEs compared with Ag nanoparticles treated groups as shown in Table 3.

Both of MNPCEs and % PCEs were statistically affected ( $p < 0.001$ ) by the Ag nanoparticles doses in groups treated with either Ag alone ( $F = 149.53, F = 110.01$ ) or CdCl<sub>2</sub> ( $F = 104.76, F = 172.93$ ), respectively. Strong positive and negative correlations between the Ag nanoparticles doses and MNPCEs and % PCEs, respectively, were indicated by the regression analysis curves (Figure 4) in all Ag nanoparticles treated groups.

**3.5. Comet Assay Results.** Results of comet assay are summarized in Tables 4(a), 4(b), and 4(c) and Figure 5 that showed representative photo for the observed various grades of DNA damage regardless of organs and treatment. Groups which received single injection of either CdCl<sub>2</sub> (1.5 mg/kg) or Ag nanoparticles in different doses showed statistical significant increases ( $p < 0.001$ ) in tail length, % DNA in tail, and tail

moment compared with the negative control group. However, CdCl<sub>2</sub> cotreatment enhanced the Ag nanoparticles induced DNA damage as shown by the statistical significant increases in tail length, % DNA in tail, and tail moment in groups injected with both CdCl<sub>2</sub> and Ag nanoparticles compared with the Ag nanoparticles treated groups' levels. This DNA damage induction was statistically affected ( $p < 0.001$ ) by the Ag nanoparticles dose as shown by one way ANOVA analysis (Tables 4(a), 4(b), and 4(c)) and regression analysis curves evidenced the strong positive correlations between DNA damage inductions and Ag nanoparticles doses in all Ag treated groups either alone or with CdCl<sub>2</sub> (Figure 6).

**3.6. MDA Level and CAT Activity.** Single i.p. injection of either CdCl<sub>2</sub> (1.5 mg/kg) or Ag nanoparticles (20, 41, and 82 mg/kg) caused statistical significant ( $p < 0.001$ ) elevations in the MDA level and decreases in CAT activity compared with the negative control level (Table 5). Furthermore, CdCl<sub>2</sub> coinjected with the different doses of Ag nanoparticles resulted in statistical significant ( $p < 0.001$ ) increases in

TABLE 4: (a) Tail length, % DNA in tail, and tail moment in bone marrow cells of mice injected i.p. with Ag nanoparticles or/and CaCl<sub>2</sub>. (b) Tail length, % DNA in tail, and tail moment in testis of mice injected i.p. with Ag nanoparticles or/and CaCl<sub>2</sub>. (c) Tail length, % DNA in tail, and tail moment in sperms of mice injected i.p. with Ag nanoparticles or/and CaCl<sub>2</sub>.

(a)

Group	Treatment (dose mg/kg)	Tail length (px)	% DNA in tail	Tail moment
1	Negative control	7.60 ± 0.98	16.57 ± 1.33	1.29 ± 0.19
2	Cd <sub>(1.6)</sub>	25.30 ± 2.54 <sup>a</sup>	28.25 ± 1.58 <sup>a</sup>	7.47 ± 1.02 <sup>a</sup>
3	Ag <sub>(20)</sub>	15.50 ± 1.11 <sup>a</sup>	23.03 ± 1.94 <sup>a</sup>	3.82 ± 0.47 <sup>a</sup>
4	Ag <sub>(41)</sub>	24.77 ± 2.67 <sup>a</sup>	31.85 ± 3.74 <sup>a</sup>	8.20 ± 0.47 <sup>a</sup>
5	Ag <sub>(82)</sub>	31.62 ± 1.80 <sup>a</sup>	43.58 ± 2.44 <sup>a</sup>	13.64 ± 1.01 <sup>a</sup>
6	Ag <sub>(20)</sub> + Cd	26.67 ± 2.34 <sup>a,b</sup>	34.59 ± 2.98 <sup>a,b</sup>	9.17 ± 0.72 <sup>a,b</sup>
7	Ag <sub>(41)</sub> + Cd	39.02 ± 2.94 <sup>a,b</sup>	46.62 ± 1.67 <sup>a,b</sup>	18.15 ± 1.12 <sup>a,b</sup>
8	Ag <sub>(82)</sub> + Cd	50.02 ± 4.88 <sup>a,b</sup>	54.74 ± 3.18 <sup>a,b</sup>	27.16 ± 2.74 <sup>a,b</sup>
Groups 3, 4, and 5		$F = 84.4, p < 0.001$	$F = 67.2, p < 0.001$	$F = 194.0, p < 0.001$
Groups 6, 7, and 8		$F = 53.9, p < 0.001$	$F = 70.7, p < 0.001$	$F = 130.7, p < 0.001$

Results were expressed as mean ± SD. <sup>a</sup>Statistically significantly different from the negative control group and <sup>b</sup>statistically significantly different from the comparable nano-Ag treated group at  $p < 0.001$  using Student's *t*-test. One way ANOVA was used to test the effect of different doses of Ag nanoparticles on the tested parameters.

(b)

Group	Treatment (dose mg/kg)	Tail length (px)	% DNA in tail	Tail moment
1	Negative control	6.23 ± 0.51	14.14 ± 1.06	0.88 ± 0.11
2	Cd <sub>(1.6)</sub>	16.60 ± 2.20 <sup>a</sup>	25.11 ± 2.46 <sup>a</sup>	4.73 ± 0.96 <sup>a</sup>
3	Ag <sub>(20)</sub>	21.29 ± 2.41 <sup>a</sup>	22.11 ± 1.72 <sup>a</sup>	4.85 ± 0.99 <sup>a</sup>
4	Ag <sub>(41)</sub>	31.27 ± 3.16 <sup>a</sup>	32.99 ± 1.18 <sup>a</sup>	10.42 ± 1.30 <sup>a</sup>
5	Ag <sub>(82)</sub>	45.31 ± 0.75 <sup>a</sup>	44.79 ± 2.64 <sup>a</sup>	20.14 ± 1.47 <sup>a</sup>
6	Ag <sub>(20)</sub> + Cd	29.29 ± 2.04 <sup>a,b</sup>	33.68 ± 2.64 <sup>a,b</sup>	9.71 ± 0.57 <sup>a,b</sup>
7	Ag <sub>(41)</sub> + Cd	45.70 ± 3.56 <sup>a,b</sup>	48.45 ± 3.50 <sup>a,b</sup>	22.45 ± 3.16 <sup>a,b</sup>
8	Ag <sub>(82)</sub> + Cd	63.47 ± 3.48 <sup>a,b</sup>	71.39 ± 4.25 <sup>a,b</sup>	45.14 ± 3.03 <sup>a,b</sup>
Groups 3, 4, and 5		$F = 133.4, p < 0.001$	$F = 170.4, p < 0.001$	$F = 185.2, p < 0.001$
Groups 6, 7, and 8		$F = 151.5, p < 0.001$	$F = 145.2, p < 0.001$	$F = 248.8, p < 0.001$

Results were expressed as mean ± SD. <sup>a</sup>Statistically significantly different from the negative control group and <sup>b</sup>statistically significantly different from the comparable nano-Ag treated group at  $p < 0.001$  using Student's *t*-test. One way ANOVA was used to test the effect of different doses of Ag nanoparticles on the tested parameters.

(c)

Group	Treatment (dose mg/kg)	Tail length (px)	% DNA in tail	Tail moment
1	Negative control	7.51 ± 1.70	12.33 ± 1.33	1.01 ± 0.34
2	Cd <sub>(1.6)</sub>	21.36 ± 3.95 <sup>a***</sup>	25.17 ± 1.72 <sup>a***</sup>	5.84 ± 1.32 <sup>a***</sup>
3	Ag <sub>(20)</sub>	15.35 ± 157 <sup>a***</sup>	22.44 ± 1.67 <sup>a***</sup>	4.33 ± 1.25 <sup>a***</sup>
4	Ag <sub>(41)</sub>	22.89 ± 2.71 <sup>a***</sup>	32.84 ± 3.53 <sup>a***</sup>	7.37 ± 0.45 <sup>a***</sup>
5	Ag <sub>(82)</sub>	34.45 ± 2.95 <sup>a***</sup>	45.64 ± 3.09 <sup>a***</sup>	15.75 ± 1.89 <sup>a***</sup>
6	Ag <sub>(20)</sub> + Cd	23.65 ± 4.21 <sup>a***,b**</sup>	36.43 ± 9.79 <sup>a***,b*</sup>	7.58 ± 1.39 <sup>a***,b**</sup>
7	Ag <sub>(41)</sub> + Cd	34.05 ± 4.82 <sup>a***,b**</sup>	52.32 ± 4.15 <sup>a***,b***</sup>	17.71 ± 3.09 <sup>a***,b***</sup>
8	Ag <sub>(82)</sub> + Cd	54.92 ± 4.63 <sup>a***,b**</sup>	73.00 ± 4.48 <sup>a***,b**</sup>	37.08 ± 9.00 <sup>a***,b**</sup>
Groups 3, 4, and 5		$F = 74.9, p < 0.001$	$F = 81.6, p < 0.001$	$F = 100.5, p < 0.001$
Groups 6, 7, and 8		$F = 60.9, p < 0.001$	$F = 173.8, p < 0.001$	$F = 357.2, p < 0.001$

Results were expressed as mean ± SD. <sup>a</sup>Statistically significantly different from the negative control group and <sup>b</sup>statistically significantly different from the comparable nano-Ag treated group at \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$  using Student's *t*-test. One way ANOVA was used to test the effect of different doses of Ag nanoparticles on the tested parameters.

MDA level and decreases in CAT activity compared with Ag nanoparticles groups' values (Table 5).

One way ANOVA showed that both of MDA level and CAT activity were significantly altered by the dose

of Ag nanoparticles (Table 5) and the regression analysis curves revealed the strong positive and negative correlations between Ag nanoparticles dose and MDA level and CAT activity in all Ag treated groups (Figure 7).

TABLE 5: The MDA level and CAT activity in bone marrow, testis, and sperms of mice injected i.p. with Ag nanoparticles or/and CaCl<sub>2</sub>.

Group	Treatment (dose mg/kg)	Bone marrow		Testis		Sperms	
		MDA level (nmol/g)	CAT activity (U/g)	MDA level (nmol/g)	CAT activity (U/g)	MDA level (nmol/10 <sup>6</sup> )	CAT activity (U/10 <sup>6</sup> )
1	Negative control	29.76 ± 1.31	8.15 ± 0.52	38.05 ± 1.66	7.01 ± 0.41	2.93 ± 0.16	46.83 ± 2.36
2	Cd <sub>(1.6)</sub>	49.49 ± 1.80 <sup>a</sup>	4.21 ± 0.62 <sup>a</sup>	57.77 ± 4.27 <sup>a</sup>	3.24 ± 0.29 <sup>a</sup>	9.95 ± 0.12 <sup>a</sup>	36.09 ± 2.33 <sup>a</sup>
3	Ag <sub>(20)</sub>	47.88 ± 1.71 <sup>a</sup>	5.58 ± 0.48 <sup>a</sup>	59.17 ± 4.75 <sup>a</sup>	4.47 ± 0.38 <sup>a</sup>	6.30 ± 0.22 <sup>a</sup>	32.79 ± 1.90 <sup>a</sup>
4	Ag <sub>(41)</sub>	60.52 ± 1.12 <sup>a</sup>	4.10 ± 0.29 <sup>a</sup>	78.04 ± 5.72 <sup>a</sup>	2.98 ± 0.29 <sup>a</sup>	9.45 ± 0.12 <sup>a</sup>	26.50 ± 2.45 <sup>a</sup>
5	Ag <sub>(82)</sub>	89.78 ± 2.48 <sup>a</sup>	2.88 ± 0.28 <sup>a</sup>	107.03 ± 7.72 <sup>a</sup>	2.20 ± 0.25 <sup>a</sup>	14.17 ± 0.45 <sup>a</sup>	17.51 ± 1.64 <sup>a</sup>
6	Ag <sub>(20)</sub> + Cd	68.93 ± 3.42 <sup>a,b</sup>	4.04 ± 0.37 <sup>a,b</sup>	92.62 ± 2.60 <sup>a,b</sup>	2.57 ± 0.49 <sup>a,b</sup>	8.64 ± 0.30 <sup>a,b</sup>	24.37 ± 1.82 <sup>a,b</sup>
7	Ag <sub>(41)</sub> + Cd	87.93 ± 1.36 <sup>a,b</sup>	2.95 ± 0.32 <sup>a,b</sup>	116.55 ± 3.44 <sup>a,b</sup>	1.95 ± 0.15 <sup>a,b</sup>	12.40 ± 0.44 <sup>a,b</sup>	13.11 ± 2.19 <sup>a,b</sup>
8	Ag <sub>(82)</sub> + Cd	116.68 ± 5.82 <sup>a,b</sup>	1.99 ± 0.08 <sup>a,b</sup>	133.43 ± 2.02 <sup>a,b</sup>	1.12 ± 0.16 <sup>a,b</sup>	21.22 ± 2.79 <sup>a,b</sup>	6.86 ± 0.74 <sup>a,b</sup>
Groups 3, 4, and 5		$F = 1088.7,$ $p < 0.001$	$F = 69.7,$ $p < 0.001$	$F = 682.8,$ $p < 0.001$	$F = 67.7,$ $p < 0.001$	$F = 879.1,$ $p < 0.001$	$F = 71.8,$ $p < 0.001$
Groups 6, 7, and 8		$F = 732.3,$ $p < 0.001$	$F = 64.2,$ $p < 0.001$	$F = 277.9,$ $p < 0.001$	$F = 27.7,$ $p < 0.001$	$F = 689.8,$ $p < 0.001$	$F = 136.4,$ $p < 0.001$

Results were expressed as mean ± SD. <sup>a</sup>Statistically significantly different from the negative control group and <sup>b</sup>statistically significantly different from the comparable nano-Ag treated group at  $p < 0.001$  using Student's  $t$ -test. One way ANOVA was used to test the effect of different doses of Ag nanoparticles on the tested parameters.

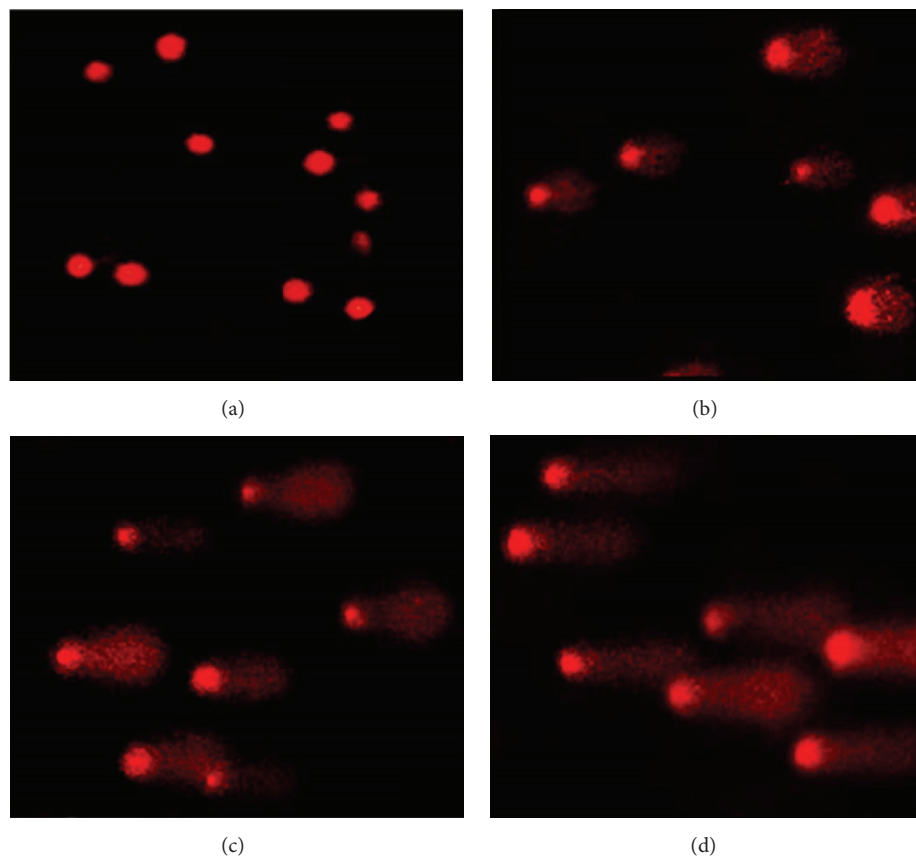


FIGURE 5: Representative photos for the observed various grades of DNA damage (b–d) and undamaged DNA (a).

#### 4. Discussion

The incredible rapid growth in the uses and applications of Ag nanoparticles because of their antimicrobial properties increases human exposure to them. On the other hand, increasing environmental pollution increases human exposure

to many other pollutants including heavy metals, for example, Cd in addition to Ag nanoparticles via various ways including contaminated water, fishes, and other sources. Therefore, it is essential to investigate the effect of Cd on the Ag nanoparticles induced genomic instability and DNA damage in mice testis, sperms, and bone marrow cells in this study.



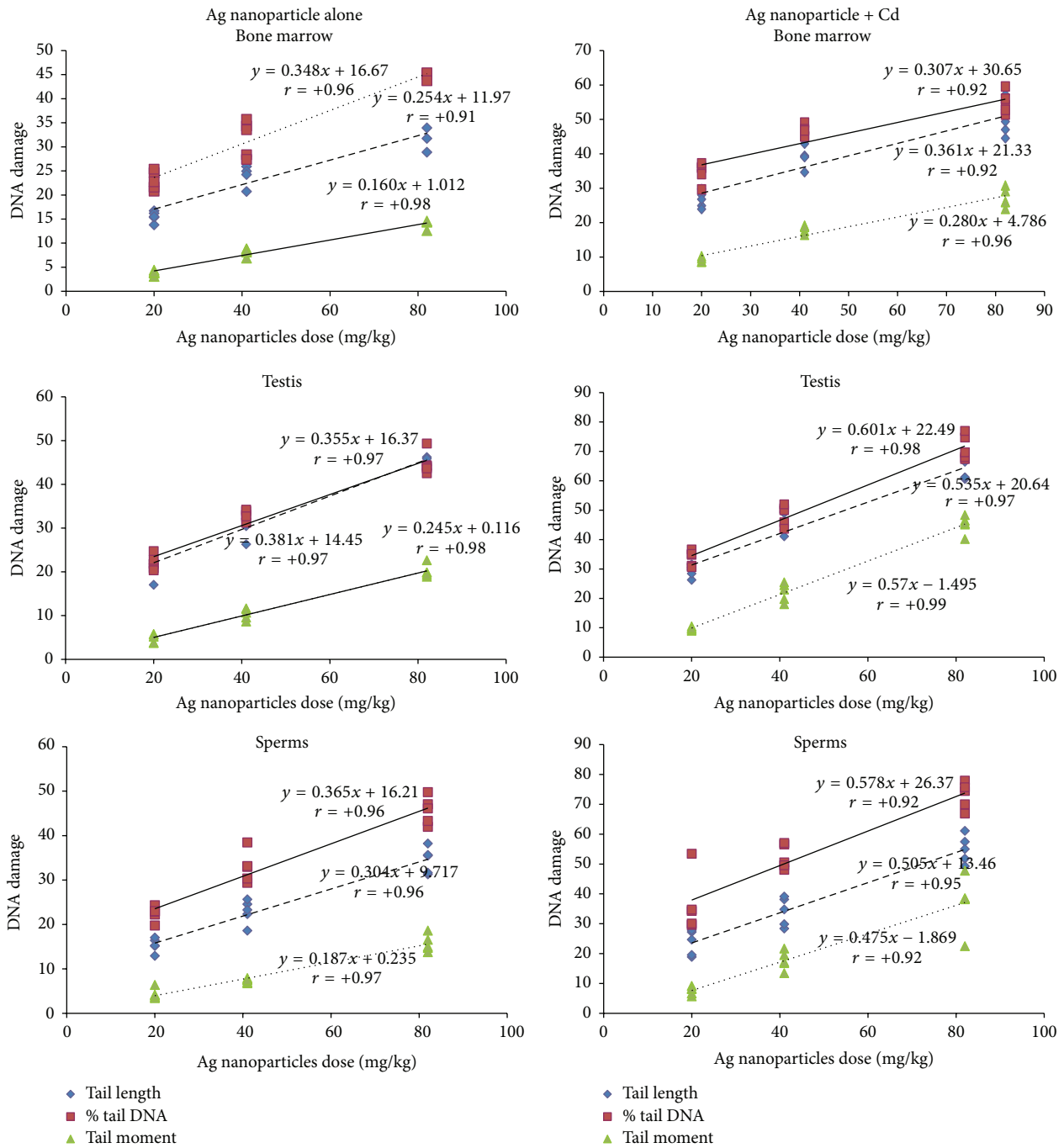


FIGURE 6: Regression lines and correlation coefficients between tail length, % DNA in tail, or tail moment and the different doses of Ag nanoparticles in mice injected i.p. with Ag nanoparticles either alone or with  $\text{CaCl}_2$ .

The observed significant elevations in sperm abnormality, MNPCs frequencies, and DNA damage inductions and significant decreases in % PCEs compared with the negative control group in this study confirmed the previously reported cyto-, clasto-, and genotoxic effects of Cd [30, 36–38]. This genotoxic effect of Cd could be attributed to its capacity to stimulate oxidative stress by interacting with the thiol groups of antioxidant enzyme and thus inhibiting them as revealed

in our study by the significant elevations in MDA level and reduced CAT activity in Cd treated group.

First our results confirmed the dose-dependent genotoxicity of Ag nanoparticles alone on both chromosomal and DNA levels by the significant elevations in micronuclei frequencies and DNA damage parameters in agreement with the previous studies [39–41]. Indeed, our finding of reproductive function deterioration in Ag nanoparticles treated male

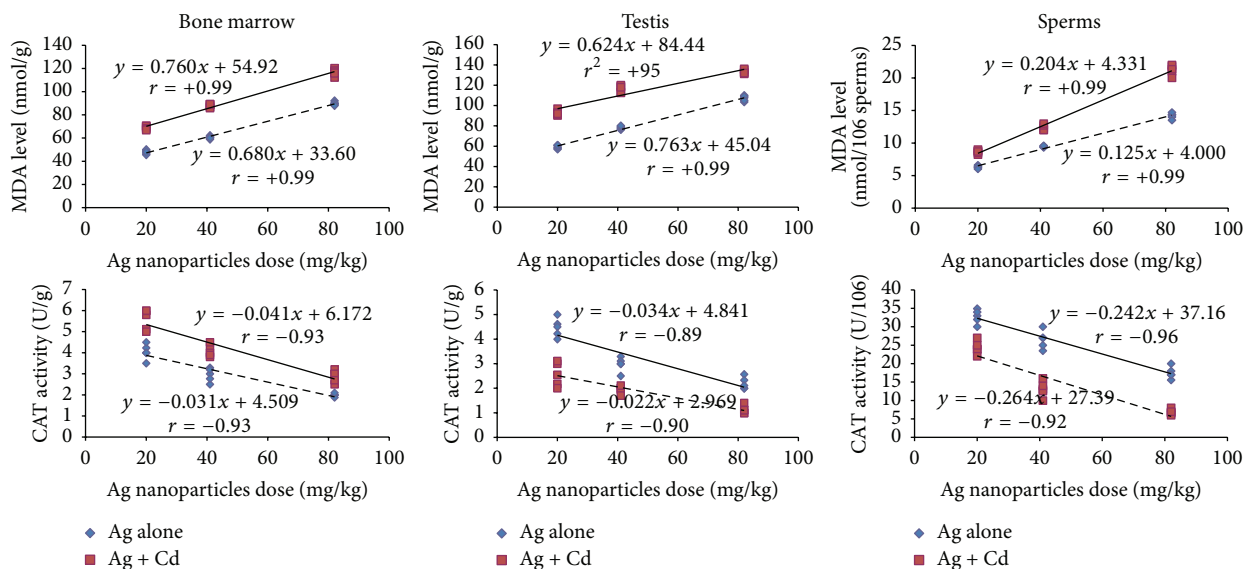


FIGURE 7: Regression lines and correlation coefficients between MDA level or CAT activity and the different doses of Ag nanoparticles in mice injected i.p. with Ag nanoparticles either alone or with  $\text{CaCl}_2$ .

mice by decreased sperm production and increased sperm abnormalities is in consistency with the previously reported impairment of sperm stability in Ag nanoparticles treated mice [24]. Hence, sperm abnormalities could be attributed to the observed nano-Ag induced chromosomal aberrations and DNA damage occurring during the packaging of genetic material in the sperms in addition to other possible genetic causes such as small deletions, point mutations, and mistakes in the spermatozoa-differentiating process during spermatogenesis.

Recently, Ag nanoparticles have been shown to damage DNA directly through the reaction of  $\text{Ag}^+$  ions released by Ag nanoparticles leading to reactive oxygen species (ROS) productions [41–43]. The ROS generation is considered as a key role in nano-Ag induced genotoxicity as increased ROS production decreases the cellular antioxidant defenses and disrupts the mitochondrial function by reacting with critical cellular molecules, such as lipids, proteins, nucleic acids, and carbohydrates, and generating additional radicals in a chain of reaction known as lipid peroxidation. In this study the dose-dependent significant elevations in MDA level and decreases in the antioxidant CAT activity revealed the ROS productions by Ag nanoparticles in accordance with previous studies that suggested oxidative stress induction as a possible mechanism for Ag nanoparticles induced genotoxicity [41, 44, 45].

On the other hand, Cd coadministration with Ag nanoparticles in different doses significantly increased the sperm abnormality, chromosomal damage, and DNA damage inductions levels compared with the Ag nanoparticles alone treated groups. Potentiating Ag nanoparticles induced toxicity could be attributed to the observed significant increases in MDA level and decreases in CAT activity revealing increased ROS generations and oxidative stress inductions by Ag nanoparticles after Cd coadministration. The ability of Cd ions to replace copper and iron in various cytoplasmic

and membrane proteins including ferritin and apoferritin resulting in elevations in the amount of free copper and iron ions that induces oxidative stress in addition to ROS mediated oxidative stress inductions by  $\text{Ag}^+$  ions thereby enhanced and potentiated the DNA damage inductions by Ag nanoparticles [41, 46–48]. Indeed, the observed significant elevations in MDA level and decreases in the CAT activity in Cd treated group revealed the oxidative stress inductions by Cd via inhibitions of the antioxidant defense system as previously mentioned in various studies [49–53].

Consequently, the observed significant elevations in Ag nanoparticles induced genotoxicity after Cd coadministration supported the previously reported cogenotoxic effects of Cd when combined with other mutagenic agents including UV-radiation, methyl methanesulfonate (MMS), and N-methyl-N-nitrosourea (MNU) [54]. Based on our data the presence of heavy metal cadmium chloride weakened cells, decreased their resistance, and increased their sensitivity to Ag nanoparticles induced chromosomal and DNA damage and thereby potentiated nano-Ag particles induced genotoxicity and increased their severity and risk.

## 5. Conclusion

The observed Ag nanoparticles induced dose-dependent sperm abnormality, clastogenicity, and genotoxicity in this study were potentiated by Cd coadministration. Indeed, Ag nanoparticles induced toxicities were strongly correlated with significant elevations in MDA level and decreases in CAT activity confirmed the consideration of oxidative stress as a possible mechanism for Ag nanoparticles induced genotoxicity.

## Competing Interests

The author declares that there are no competing interests.

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