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Evaluation of Genotoxic Effects of Silver Nanoparticles on Bone Marrow Chromosome Aberrations in Laboratory Male Albino Mice *Mus musculus*

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Abstract

Several desirable properties of silver nanoparticles (AgNPs) have found extensive use in consumer and healthcare products. Due to their potential to penetrate the nucleus and harm genetic material, their adverse effects, however, are mostly unknown and appear inevitable. This study aimed to determine genotoxic potential of AgNPs using mitotic index (MI) and structural chromosome aberrations (SCA) test in bone marrow cells of *Mus musculus* male albino mice. Two generations were experimented in this study, the first and second generation. In the first generation, five groups of five male mice including control group were used and intraperitoneally (IP) injected with two doses of AgNPs (50 mg/kg and 150mg/kg) in one-time and two-time doses manner. Then two-time dosed 150 mg/kg group was left for breeding and their male progenies (described as second-generation group) were dissected for detecting whether that abnormality in the male parent will transmit to progenies or not. Bone marrow cells were taken 24 hours following the last treatment. The results showed that AgNPs exposure significantly increased ($P \leq 0.05$) number of SCA and decreased (MI) compared to negative control. Centromere breaks and gaps, along with ring chromosomes, also were the most frequent chromosome aberrations. The results suggest that AgNPs may be able to cause SCA-mediated genotoxicity in mice, with declining MI ratio after increasing dose and injection frequency. Still regular monitoring of their possible health effects as well as further characterization of their genotoxicity is necessary.

Keywords: Silver nanoparticles, Genotoxicity, Chromosome aberrations, Mitotic index, AgNPs dose

تقييم التأثيرات السمية الجينية لجسيمات الفضة النانوية على انحرافات كروموسوم نخاع العظم في ذكور الفئران البيضاء المختبرية

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الخلاصة

وجدت العديد من الخصائص المرغوبة لجسيمات الفضة النانوية (AgNPs) استخدامًا واسعًا في المنتجات الاستهلاكية ومنتجات الرعاية الصحية ، في حين أن آثارها الضارة غير معروفة في الغالب ويبدو أنها حتمية بسبب قدرتها على اختراق النواة وإلحاق الضرر بالمواد الوراثية. تهدف هذه الدراسة إلى تحديد التأثيرات السمية لـ AgNPs على الجينات باستخدام مؤشر الانقسام (MI) والانحرافات الهيكلية للكروموسوم

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(SCA)في خلايا نخاع العظام من ذكور الفئران البيضاء *Mus musculus*. تم اختبار جيلين في هذه الدراسة: في الجيل الأول، خمس مجموعات من خمسة ذكور الفئران بما في ذلك مجموعة السيطرة وتم حقنهم داخل الصفاق (IP) بجرعتين من AgNPs (٥٠ مجم/كجم) و (١٥٠ مجم/كجم) بجرعة لمرة واحدة و مرتين. جرعة مرتين من مجموعة (١٥٠ مجم/كجم،) تُركت للتكاثر وتشريح أسلافها الذكور (الموصوفة بمجموعة الجيل الثاني) للكشف عما إذا كانت هذه التشوهات في الوالد الذكر ستنتقل إلى النسل أم لا. بعد ٢٤ ساعةمن المعاملة بجسيمات الفضة النانوية،تم أخذ خلايا نخاع العظم،لوحظ ان هناك زيادةمعنوية($P\leq0.05$)في التغييرات الكروموسومية(SCA) وانخفاض معامل الانقسام الخلوي(MI) مقارنة مع معاملة السيطرة. centromere breaks, centromere gaps, ring chromosomesكانت أيضًا أكثر الانحرافات الصبغية شيوعًا. تشير النتائج أن AgNPs قد تكون قادرة على إحداث سمية وراثية بوساطة احداث تغييرات كروموسومية في الفئران، مع انخفاض نسبة (MI) بعد زيادة الجرعة وتكرار الحقن. لا يزال الرصد المنتظم لآثارها الصحية المحتملة كذلك مزيد من توصيف سميتها الجينية أمر ضروري.

1. Introduction

Silver nanoparticles (AgNPs) being one of the most commonly used NPs, are best known for their usage in biological applications (antibacterial, drug-delivery systems, dental applications, wound, and bone healing, etc.) [1, 2], cosmetics (shampoo, soap, toothpaste, intimate body care products, face masks, and make-up)[3] and in the treatment variety of diseases (malaria, lupus, tuberculosis, typhoid, and tetanus)[4]. Despite their wide range of utility, AgNPs impacts on human health and the mechanisms of action are not fully understood. To accurately assess the risks to humans, it is crucial to investigate their potential toxicity in living organisms, particularly in mammals [5]. In addition, there is a growing concern over AgNPs detrimental effects on both human health and the environment [6]. To date, the studies that report on the toxic effects of AgNPs either *in vitro* [7-9] or *in vivo* [6, 10-13] further provide data indicating adverse effects on cells exposed to AgNPs. Nanoparticles that can enter the body through the skin, lungs or digestive system and induce a variety of toxicological effects, are likely to persist for a very long time in the body and the ecosystem [14]. Several *in vivo* studies have reported that silver nanoparticles cause a reduction in mitotic index proportion [10]. Also other researchers have revealed that mitotic index, cell division and chromosome performance of *Allium cepa* obviously changed after treatment with different Ag concentrations in different duration periods [15]. However, other authors have reported that AgNP-mediated production of chromosomal aberrations (CA) plays a significant role in genotoxicity [10]. Similarly *in vitro* studies have showed impairment in DNA synthesis, micronucleus formation and DNA breakage after exposure to AgNPs [8]. Additional *in vivo* investigations have also supported that AgNPs induce SCA such as chromatid, chromosome gaps and breaks, and acentric fragments formation, due to detectable rise in DNA damage in rats or mice bone marrow leukocytes [6, 10, 11]. While a study by P. Debnath *et al.* [12] reported that AgNPs between 25-40nm particle size of high concentration can cause much lower chromosomal aberrations (fragments, lagging chromosome bridges, and stickiness) when treated on *A. cepa* roots.

There aren't many reports on AgNPs genotoxicity, especially when it comes to *in vivo* effects. Additionally, the current findings of investigations on the genotoxicity of AgNPs are conflicting for some reasons: nanoparticle size, concentration and surface functionalization all affect how harmful they are.It was reported that cytotoxicity, inflammation and genotoxicity are all significantly influenced by the sizes of AgNPs [16, 17]. The induced modification will be passed on to subsequent cell generations and could eventually result in diseases such as cancer [18]. Therefore, AgNPs immediate effects are unknown. This study was a step towards understanding the genotoxic potential of AgNPs as it aimed to investigate the genotoxicity of

silver nanoparticles at different doses and recurrent administration to the bone marrow cells in albino male mice *Mus musculus.*

2. Materials and methods

2.1 Nanoparticle

Silver nanoparticles (AgNPs) were obtained from the US Research Nanomaterials company (USA). The physical characteristics of the particles according to manufacturers' data were 20nm in diameter and 99.9% purity in trace metal basis.

2.2 Characterization of Silver Nanoparticle (AgNPs)

AgNPs were characterized by using: (1). Transmission electron microscopy (TEM) to confirm manufacture's information about particle size. (2). Scanning electron microscopy (SEM) micrographs, according to the SEM AgNPs were almost spherical but could aggregate inside the solution. (3). X-ray diffraction analysis (XRD) to supply information about crystallographic structure of the nanoparticle also detecting its purity. (4). Zeta potential test was also used to find stability of silver NP, regarding the results of material quality was good (Figure 1 & 2).

2.3 Nanoparticle's Solution Preparation

AgNPs powder was suspended directly in double deionized water and dispersed by ultrasonic vibration (100 W, 30 kHz) for 30 min to produce low dose of 50mg/kg and high dose of 150mg/kg stock solution, and then daily sonication was used for only 10 min before each experiment to prevent precipitation and aggregation.

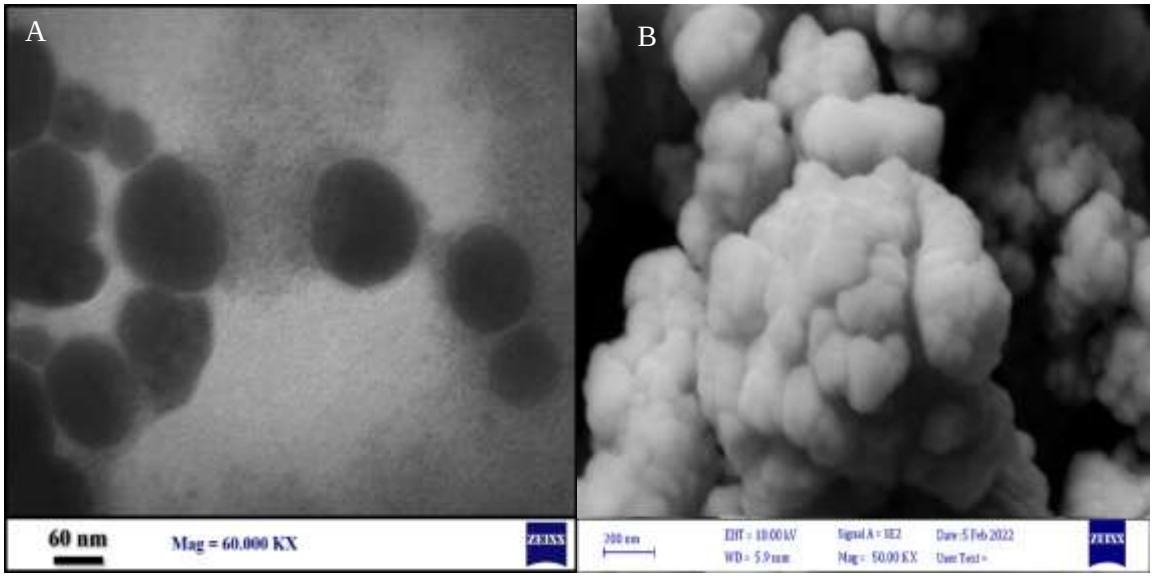

Figure1: (A) Transmission electron microscopy (TEM) image of silver nanoparticles (AgNPs); scale bars indicate 60 nm.
(B) Scanning electron microscopy (SEM) image of silver nanoparticles (AgNPs); bars indicate 200 nm. .

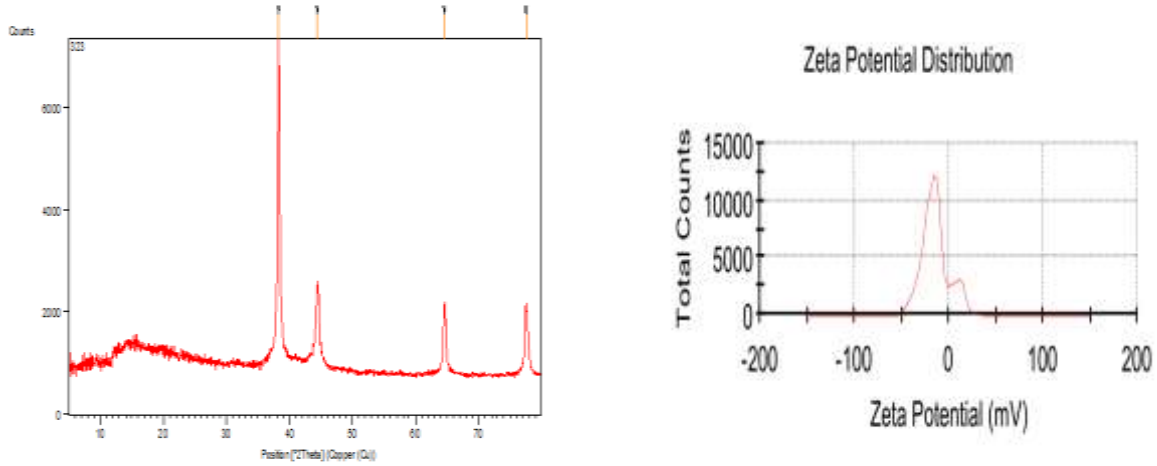

Figure 2: (A) X-ray diffraction test that gives informative data about purity of the AgNPs. (B) Zeta potential analysis to display stability of AgNPs.

2.4 Doses of Silver Nanoparticles

Groups of five male mice each were treated with two different AgNP doses in first generation.They were intraperitoneally (IP) injected using insulin syringes at doses of 50 and 150 mg/kg. One dose per 24h was given for one time dose and two doses per 48h were given for two-time doses with 24h intervals between them.Later the animals were dissected after 24h of administration. Deionized water was recommended as negative control. Doses were chosen based on previous research in the related area [10]. Second generation G2 group wasn't injected with any dose, only deionized water was used to balance phycological distress in other groups.

2.5 Animal Grouping and Dosing

Thirty-three albino male mice were used in this study. They were divided into five groups of five male mice for the first generation (G1) and one group of eight mice for the second generation (G2). In G1, 4 treatment groups (T1, T2, T3 and T4) with control group were examined. While in G2, only one treatment group (T5) was tested. Animal dosing was designed as following:

Control: Mice were treated only with distilled water in accordance withthe mouse body weight.

T1 (L/1): Mice were treated with a single-dosage of silver nano solution at a low dose of 50 mg/kg.

T2 (L/2): Mice were treated with a double-dosage of nano solution at a low dose of 50 mg/kg. One dose per day injected.

T3 (H/1): Mice were treated with a single-dosage of nano solution at a high dose of 150 mg/kg.

T4 (H/2): Mice were treated with a double-dosage of nano solution at a high dose of 150 mg/kg. One dose per day injected.

T5: Mice were not treated with any NPs dose but were only injected with DWas they were progenies of (T4) males breeding.

2.6 Animal Study

This study was approved by the local Ethical Committee for Animal Experimentation of the Department of Biology, College of Science-University of Sulaimani (permission 002, 2-

August-2021) based on CONCEA (National AnimalExperiment Control Council) ethical norms for animal experimentation.

2.7 Structural ChromosomeAberrations Assay (SCA)

According to Clendenin (1969) the animals were injected intraperitoneally with colchicine solution adjusted to mouse body weight (4 mg/kg) for about 4 hours before dissection [19]. The mice were sacrificed by cervical dislocation. Femora were removed, bone marrow was extracted by flushing 5 ml of (normal saline solution 0.9%) and collected in a tube, and then centrifuged at 1000 rpm for 10 minutes. The supernatant was removed and 5m1 of hypotonic solution KCL0.75 M was added slowly and mixed thoroughly, and then the mixture was put in water bath for 20 min at 37°C which was later centrifuged at 1000rpm for 10 min. The supernatant was discarded. Total of 11 ml of fixative Methanol: Acetic acid 3:1 volume was added in 3 steps (1, 5and 5ml respectively) with 3-times centrifugation in each step after fixative addition. Finally, 3-5 drops of cell suspensions were dropped from 50cm height onto a clean moist slide, and then the slide was air-dried at room temperature, stained with Giemsa for 30 minutes, washed with tap water. At least 100 metaphase cells per animal were scored to indicate SCA [20].

2.8 Mitotic Index

The mitotic index (number of dividing cells/total number of cells scored×100) was used to determine the rate of cell division [21]. The slides prepared for the chromosome aberrations assessment were also used for measuring the mitotic index. Randomly chosen views on the slides were observed to count the number of dividing cells (metaphase stage) and the overall number of cells. At least 1000 cells were examined per animal by using light microscope with objective lens 100X for scoring MI and also SCA.

2.9 Statistical Analysis

All data was expressed as means ± S.E. Statistical significance of differences among different groups, was evaluated by one-way analysis of variance (ANOVA) and unpaired student T-test followed by Tukey test for multiple comparisons. A *P-value*≤0.05 was considered statistically significant.

3. Results

3.1 Mitotic Index

The mitotic index (MI) was used to determine the rate of cell division. According to the results of G1, the MI ratio decreased as the silver nanoparticle dose was increased. Mitotic indices of 22.94±0.3970%, 21.02±0.6192%, 19.82±0.3484%, 18.32±0.2177% and 17.96±0.4833% were recorded for the control T1, T2, T3 and T4 group respectively (Table 1). According to the data analysis, a highly significant difference(*P≤0.05*) existed between T1 (21.02±0.6192) & T4 (17.96±0.4833) and also between T2 (19.82±0.3484) and T4 decreasingly. Whereas, significant decrease (*P*≤0.05) occurred among T1 (21.02±0.6192) and T3 (18.32±0.2177). Expectedly, a significant decrease (*P*≤0.05) existed between control (22.94±0.3970) with all remaining treated groups in a dose-dependent and injection-frequency dependent manner. MI ratio among second-generation progenies G2 showed an extremely significant difference (*P*≤0.001) between T4 (17.96±0.4833) and T5 (29.06±0.5362) increasingly meaning several effects of double-dosage / high dose AgNPs in G1 parents. These effects, however, didn't transmit to G2 progenies (Table 2).

3.2 Structural ChromosomeAberrations (SCA)

Structural chromosome aberrations (SCA) include many aberration types. Some of them in this study were considered noticeable and were measured under microscope examination. Results of the first generation showed that silver nanoparticle stimulated a dose-dependent genotoxic effect between control, low-dose and high-dose treatments. Among all tested aberration types, ring chromosomes, centromere break and centromere gap increased in a dose dependent and administration-frequency dependent manner which was also noted to be more frequent than others. However, chromatid break and acentric fragments occurred less frequently. Additionally, number of normal metaphases dose-dependency decreased (Table 3). In the second generation, all previous aberration types were also measured. The comparison was conducted between double-dosage high dose group T4 in G1 and their non-dosed progenies T5 in G2 (Table 4). According to the comparison results, it can be said that all aberrant ratios measured in G1 were about twofold percentage as compared to G2. So, it is clear that using high dose / 2times in the first generation worked more severely and toxicity effect of AgNPs in male parents didn't transmit to second generation progenies to induce anomalies in chromosomes.

Table 1: Effects of low dose and high dose AgNPs on MI ratio in bone marrow cells

Treatments	Mean ±S.E. of Mitotic Index Ratio.
Control	22.94±0.3970 [a]
L/1(T1)	21.02±0.6192 [b]
L/2(T2)	19.82±0.3484 [b]
H/1(T3)	18.32±0.2177 [c]
H/2(T4)	17.96±0.4833 [c]

Note: Using Tukey test analysis, the metaphase cells that have been observed by microscopic examination were calculated among treatment groups. L=low dose, H=high dose (1=one time administration, 2=two times administration), letters (a and b) = represent significant difference value ($p \leq 0.05$). Same letter= no significant difference, different letter=significant difference.

Table 2: Effects of double-dosed high dose AgNPs and non-dosed treatments in G2 on MI ratio in bone marrow cells

Treatments	Mean ±S.E. of Mitotic Index Ratio.
T4	17.96±0.4833 [a]
T5	29.06±0.5362 [b]

Note:Using T-test analysis, the metaphase cells have been observed by microscopic examination and calculated. T4= high dose/2 times treatment, T5= non-dosed G2 progeny treatment. ($P \leq 0.001$) was used to compare treatment groups.

Table 3: Effects of low dose and high dose AgNPs on SCA in bone marrow cells Mean ±S.E

Aberrant / Treatments	Normal Metaphase	Ring Chromosome	Chromatid Gap	Chromatid Break	Centromere Break	Centromere Gap	Dicentric	Acentric
Control	71.80± 1.020 ^{a}	9.400± 0.4000 ^{a}	5.400± 0.6000 ^{a}	3.200± 0.5831 ^{a}	5.000± 0.547^{a}	4.000± 0.3162 ^{a}	6.200± 0.374^{a}	1.000± 0.3162^{a}
L/1 (T1)	59.20± 2.458 ^{b}	10.80± 0.5831 ^{a}	5.600± 0.6782 ^{a}	4.600± 0.7483^{ab}	8.600± 0.678^{b}	6.000± 0.3162 ^{b}	6.200± 0.374^{a}	2.600± 0.2449^{b}
L/2 (T2)	44.20± 3.382 ^{c}	16.60± 0.9798 ^{b}	6.800± 0.9695^{ab}	6.000± 1.140 ^{ab}	11.60± 0.600^{c}	8.000± 0.4472 ^{c}	6.400± 0.509^{a}	3.600± 0.4000^{b}
H/1 (T3)	40.60± 1.327 ^{c}	23.20± 1.241 ^{c}	8.600± 0.8124^{ab}	6.800± 1.020 ^{b}	12.20± 0.583^{c}	8.200± 0.3742 ^{c}	7.200± 0.489 ^{a}	3.000± 0.3162^{b}
H/2 (T4)	39.20± 2.782 ^{c}	29.60± 1.166 ^{d}	9.800± 1.114 ^{b}	6.400± 0.4000^{ab}	9.800± 0.3742 ^{d}	10.20± 0.3742 ^{d}	9.600± 0.7483 ^{b}	3.600± 0.4000 ^{b}

Note: Using Tukey test Analysis, the SCA has been observed by microscopic examination were calculated among the treatment groups, letters (a and b) represent significant difference value ($p \leq 0.05$), same letter= no significant difference, different letter= significant difference.

Table 4: Effects of double-dosed high dose AgNPs and non-dosed treatment in G2 on SCA in bone marrow cells
Mean ±S.E.

Aberrant / Treatments	Normal Metaphase	Ring Chromosome	Chromatid Gap	Chromatid Break	Centromere Break	Centromere Gap	Dicentric	Acentric
T4	39.20± 2.782 ^{a}	29.60± 1.166 ^{a}	9.800± 1.114 ^{a}	6.400± 0.4000 ^{a}	9.800± 0.3742 ^{a}	10.20± 0.374 ^{a}	9.600± 0.748 ^{a}	3.600± 0.4000 ^{a}
T5	75.75± 1.359 ^{b}	8.500± 0.6814 ^{b}	4.500± 0.4629 ^{b}	4.000± 0.2673 ^{b}	4.375± 0.1830 ^{b}	3.125± 0.350 ^{b}	3.375± 0.375 ^{b}	3.000± 0.2673 ^{a}

Note: Using unpaired T-test analysis, the SCA has been observed by microscopic examination and calculated among the treatment groups. T4= high dose/2 times, T5= non-dosed G2 progeny. Letters a and b represent significant difference value ($p \leq 0.05$), same letter=non-significant difference, different letter= significant difference.

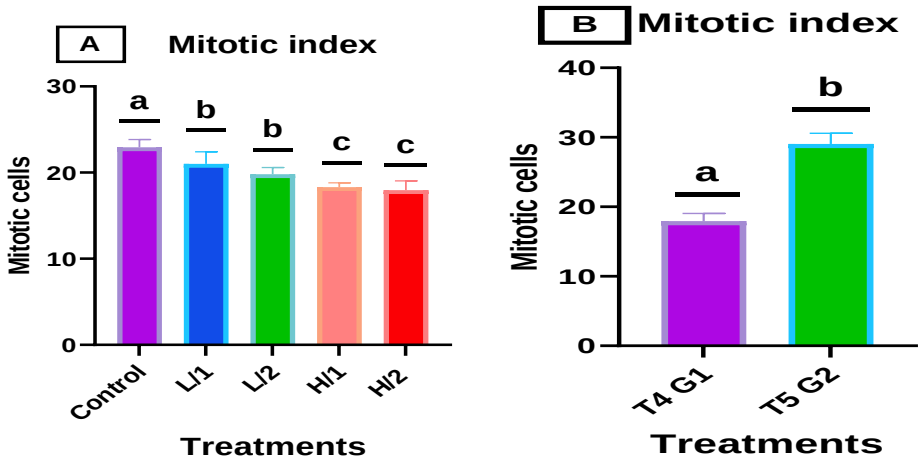

Figure 3: (A) Bar graph of mitotic index among treated groups in G1. (B) Bar graph of mitotic index among treated groups in G1 and G2.

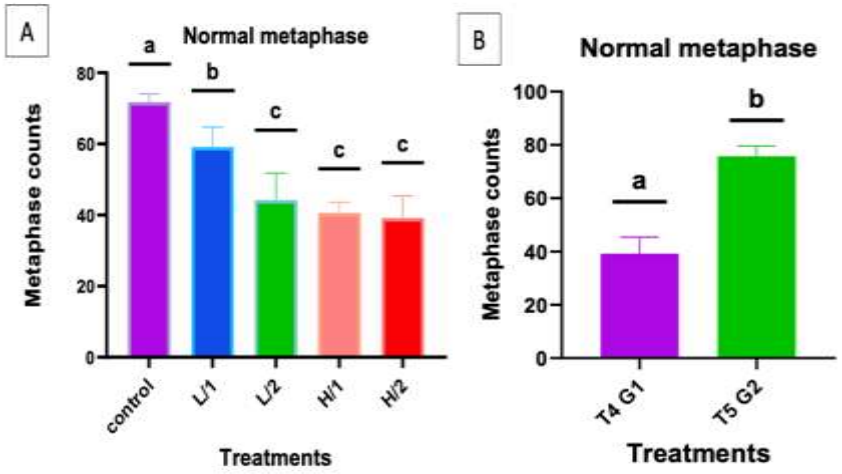

Figure 4: (A) Bar graph of normal metaphase among treated groups in G1. (B) Bar graph of normal metaphase among treated groups in G1 and G2.

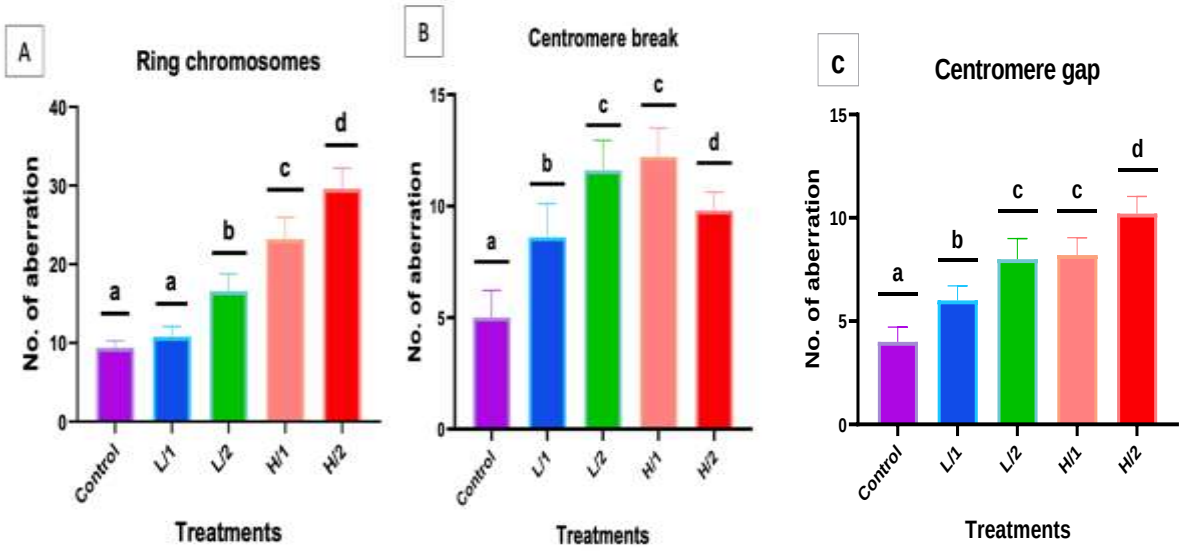

Figure 5: (A) Bar graph of ring chromosome aberration among treated groups in G1. (B) Bar graph of centromere break among treated groups in G1. (C) Bar graph of centromere gap among treated groups in G1.

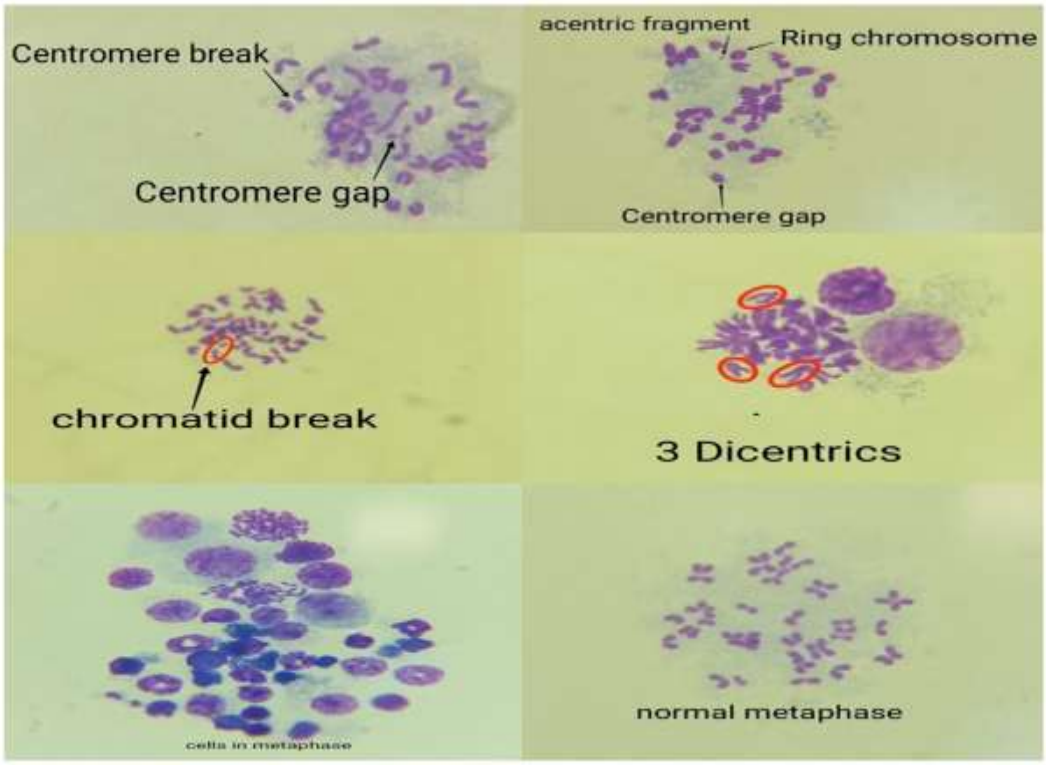

Figure 6: Types of structural chromosome aberrations (SCA) induced by silver nanoparticles 20nm particle size (1000X), with representing some cells in metaphase

4. Discussion

The metaphase analysis of bone marrow cells discovered several types of chromosome aberrations which included chromatid and chromosomal breaks, gaps, unions and fragments. In a study performed on Sprague Dawley rats 10nm silver nanoparticles in different doses were administered orally. The experimental data suggested that chromatid gaps and breaks were distinguished to be more common than other aberration forms[10]. However, in this study centromere gaps and breaks, along with ring chromosomes, were more prominent than others. The incidence of chromosomal aberrations (CA) also enlarged with raising doses of AgNPs. It was a dose-dependent increase in genotoxic effects of AgNPs, as for this study, similar outcomes were achieved. Results of [6] do not resemble this present research as the comet assay results did not reveal a detectable rise in DNA damage in rat bone marrow myeloblast, when rats were injected intravenously with a single dose of 5 mg/kg of AgNPs with 20 nm particle size. These findings might be a result of leukocyte genetic material being resistant to Ag nanoparticle's toxicity. As the first sample was taken at 24 h after treatment, it is also possible that quick reparation of DNA damage might have happened.

In a recent study when Ag^{+} and AgNPs with average particle size between 27-106 nm were administrated intravenously (IV) to Sprague-Dawley rats for 24 h, a chromosome aberration occurred due to Ag^{+} and AgNPs effectiveness [11]. In an Indian study coordinated in 2018, to assess efficacy of both silver and gold nanoparticles on the chromosomal aberration in *Allium cepa* roots, statistical results observed that AgNPs between 25-40nm particles size of high concentration (10 mg L^{-1}) showed much lower chromosomal aberration in comparison to AuNP with respect to control group. So perhaps toxicity of AuNP may be higher than AgNP. [12]

Findings of the current study are in accordance with those of AshaRani *et al.* [7] who found that human fibroblast cells exposed to AgNPs displayed comparable chromosomal abnormalities. This is because electron micrographs showed that AgNPs were uniformly distributed inside each cell, both in the cytoplasm and the nucleus. Human cells exposed to AgNPs demonstrated chromosomal instability and mitotic arrest. Also Dobrzyńska *et al*. reported that 20 nm AgNPs exposed polychromatic erythrocytes of bone marrow cells were the main target of nanoparticles. They also discovered that AgNPs showed a higher impact on genetic damage than other nanomaterials in these cells [6]. Study of Asharani *et al*. used normal human lung fibroblast (IMR-90) cells that were exposed to different dosages of silver nanoparticles *in vitro*. Cells treated with AgNPs were carefully evaluated for chromosome abnormalities. In the study, treated cells were exposed to three concentrations of nanoparticles. The two largest concentrations of AgNPs (50 μg/mL and 100 μg/mL) increased the frequency of structural chromosomal abnormalities, frequency of micronucleus, DNA damage and decreased the mitotic index. However, fibroblasts treated with the lower concentration (25 μg/mL) only increased centric and acentric fragments. The results confirmed that AgNPs were endocytosed by the cells and were present in the nuclei. DNA synthesis and damage, and chromosome segregation, may all be affected by such an occurrence [8]. Numerous *in vitro* and *in vivo* investigations have established that AgNPs with smaller particle sizes are more genotoxic than any other nanoparticles. AgNPs might easily diffuse into the nucleus through the pores because of their small size [6, 22-26]. However, further experimentations are still required to investigate whether small-size AgNPs cause more genetic impairment andoxidative stress than larger ones [27, 28]. Regarding viability test assessment, research performed by Fuster *et al.*[9] in which silver NPs capped with citrate and used on Human T98 glioblastoma cells, were almost resistant to the cytotoxicity induced by AgNPs. The exposure to 40 μg/ml AgNPs for 72 hours diminished cell viability by only 5%. It can, therefore, barely reduce cell viability. Several authors informed that some of the effects related to AgNPs might be partially associated to soluble silver ions (Ag^{+}) released throughout exposure [29, 30].

Finally in recent *in vitro* and *in vivo* genotoxicity investigations, numerous parameters, including physio-chemical properties and experimental settings, were used to detect the genotoxic response. However, findings of current results need more other cytogenetic techniques like micronucleus assay, ROS detection in bone marrow cells and comet assay to evaluate the genotoxicity of Ag nanoparticles in bone marrow cells of the mice more accurately.

5. Conclusion

In the first generation tested group, AgNPs reduced the total ratio of mitotic index (MI) of the metaphase of the treated animals in a dose dependent manner. While in second generation, total MI percentage showed highly significant differences and the effects of AgNPs worked more profoundly in G1. These effects, however, did not pass on to G2 and rate of cell division remained normal. Generally, in the first generation SCA proportion increased in a dose-dependent and injection-frequency dependent manner and numerous chromosome aberrations including centromere break and gap, chromatid break and gap, ring chromosome, dicentric and acentric fragments, which were formed in all treated groups. However, ring chromosomes, centromere breaks and centromere gaps were more prominent than others. Additionally, number of normal metaphases dose-dependently decreased. Second generation group showed a highly significant difference with the first generation in enhancing SCA and it is totally safe like in a control group and rich in normal chromosomes.

Conflict of Interest

The authors declare no conflict of interest.

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