Original Article

Silver Nanoparticles Genotoxicity on Normal Human Melanocyte Cell Line (HBF-4 cells).

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Abstract:

Background:Nanotechnology has been rapidly used in diverse consumer products in both medical and industrial fields; which has raised various concerns. Silver nanoparticles (AgNPs) are one of the most widely used nanomaterials due to their excellent broad-spectrum antibacterial properties. However the studies on their cyto- and geno-toxic effects are scarce. The present study aim to evaluate the DNA damaging effects of AgNPs and estimate the release of intracellular reactive oxygen species (ROS) on HBF-4 normal human melanocyte cells. **Methods**: The study was conducted on HBF-4 cells, they will be treated with AgNPs at different levels (0.01, 0.1, 1, 10 and 100 µg/mL), LC50 was determined. Cells were then divided into two groups: the first was treated with the LC50 dose and the second was treated with 0.1 LC50 dose. DNA fragmentation assay using diphenylamine (DPA) technique and Intracellular ROS assay were measured and compared to control group. **Results**: Significant increases in DNA damage and release of intracellular ROS were observed on treated HBF-4 cells with LC50 and its 1/10 LC50 value in comparison to the control group. **Conclusion**: silver nanoparticles can induce oxidative stress and DNA damage in normal human melanocyte cells with a potential carcinogenic effect to be considered. Further investigations form their genetic alterations mechanisms are required.

Keywords: Silver nanoparticles - HBF-4 epithelial cells - DNA fragmentation - Intracellular ROS.

I. INTRODUCTION:

Nanoparticles (NPs) are functional materials characterized by their unique physicochemical properties such as small size, greater surface area compared to their volume as well as their different shapes and electronic, magnetic, optical, mechanical properties, which allow them to be implicated in diverse industrial and biomedical applications especially consumer products such as clothing, sporting goods, personal care products, cosmetics and sunscreens. Nevertheless, there are various concern regarding the knowledge on their toxicity **(**Gurunathan et al., 2018).

Silver nanoparticles (AgNPs) are nano-materials used excessively in many industrial and medical products due to their excellent broad-spectrum antibacterial properties; however, there is a growing concern for their potential adverse effects (Chen et al., 2019).

The adverse health effects of toxic nanoparticles are much linked to production of reactive oxygen species (ROS) and subsequently states of cellular oxidative stress. Thus, redox dependent processes and their effects of ambient engineered nanoparticles have become the main point of research in the field (Sthijns et al., 2017).

Silver (Ag) has been declared as Group D potential carcinogen according to the Integrated Risk Information System. Moreover, Ag nanoscales are known to induce high reactivity owing to their physical characters which may cause DNA damage. Therefore, it is possible to be implicated in the first step of carcinogenesis induction (Choo, 2017).

As further as we know, the studies conducted to investigate both cytotoxicity and genotoxicity of metallic nanoparticles on normal untransformed human cells with a stable diploid karyotype are scant. Therefore, there is pressing necessity to study the mechanisms of action of nanoparticles on untransformed normal cells (Franchi et al., 2015).

The present study aimed to evaluate the cytotoxic DNA damaging effects of AgNPs and estimate the release of intracellular ROS on normal human melanocyte cell line HBF-4 cells.

II. MATERIAL AND METHODS

Otherwise stated, all material were purchased from Sigma Aldrich, Egypt.

II.1. Nanoparticle suspension: **synthesis** and **preparation:**

The silver colloid was synthesis by using modified Ismail et al. method.

By coprecipitation with ultrasonic assistant, silver nitrate and trisodium citrate have been used as precursor and reducing material for the synthesis of AgNPs. In typical synthesis method, 100 ml of 0.001M $AgNO_3$ was heated to boil then put in ultrasonic prop device at condition of cycle 50 and amplified 70% with add drop by drop from solution of 0.1M trisodium citrate until change of color was obtain (pale yellow) then removed from the ultrasonic device and waiting until cooled to room temperature in dark area to avoid lights (**Ismail et al ., 2020).**

II.2. Nanoparticle characterization:

XRD (D8 Discovery-Bruker Company) at condition of 40 KV and 40 AM (1600W) at speed scan 0.01 and 2(θ) range from 10° to 80°. Microscopic properties was carried out by transmission electron microscope (TEM) model EM-2100 High-Resolution at magnification 25X and voltage 200 Kv. Zeta potential were carried out by instrument manufacture by Malvern instruments Ltd. Model of Nano Sight NS500 to determine the size and zeta potential of nanoparticles (Zhang et al., 2016).

II.3. Normal Human Melanocyte Cell Line (HBF-4 cells) culture preparation:

Normal human melanocyte cell line (HFB4) was purchased from the American Type Culture Collection (USA). According to manufacturer instructions, cells were allowed to replicate in 75cm^2 cell culture flasks using RPMI-1640 medium and DMEM, respectively supplemented with 10% (v/v) fetal bovine serum and incubated in 5% (v/v) CO_2 incubator at 37°C. 0.25% (w/v) trypsin solution and 0.05% (v/v) EDTA were added for 5 min to detach confluent cells. The the detached cells were centrifuged using cooling centrifuge (FIRLABO,france). Cell pellets were re-suspended in growth medium; then they were plated in 96-well cell culture plates at a concentration of 2×10^5 cells/ml, then incubated at 37 °C for 24 using NUVE,EN-120,turkey incubator**.**

II.4. Cytotoxicity assessment

Cytotoxicity assessment was performed in dark environment using freshly prepared 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for viability assay to explore the cytotoxicity of the prepared AgNPs. Cells were cultured at 1×10^5 density on 96-well plates after adding 20 ml MTT solution, then were left incubated overnight in humidified air with 5% CO_2 at 37°C to allow cell adhesion at

the plate bottom. The aliquot was removed by adding 200ml acidic isopropanol and mixed well. The medium was replaced with a new one containing different concentrations of AgNPs (0.01, 0.1, 1, 10 and 100 µg/mL in medium, respectively) for 24h. The medium containing AgNPs was then removed, cells were washed with phosphate-buffered saline (PBS), and MTT reagent was then incubated for additional one hr in 37^{o}C in dark. After incubation with MTT, cells were washed again with PBS and then dimethyl sulfoxide was added to solubilize the produced insoluble formazan. Absorbance was measured at 570 nm (background wavelength is 630nm) using an ELIZA microtiter plate reader (BIOTEK ELX-800,USA).

II.5. DNA fragmentation.

According to manufacturer instructions, 1.0 ml of cell suspension ($5x10^5$) were inserted in labeled tubes in complete RPMI medium; centrifuged at 200xg at 4°C for 10 min. Then, supernatants were carefully transferred in new labeled tubes. Added 1.0 ml TTE solution (TE buffer pH 7.4 with 0.2% Triton X-100, stored at 4°C) and 1.0 ml of 25% Trichloroacetic acid (TCA) to the tubes containing the pellets, and vortexd vigorously. Allow precipitation to proceed overnight at 4°C, then recover precipitated DNA by pelleting for 10 min at 20,000xg at 4°C. Supernatants were discard by aspiration. DNA was hydrolyzed by adding 160 ml of 5% TCA to each pellet and heating 15 min at 90°C in a heating block. Then a blank with 160 ml of 5% TCA alone was prepared. For each tube 320 ml of freshly prepared DPA solution were added, then vortex to allow color to develop for about 4 h at 37°C or overnight at room temperature. Two 200 ml aliquots of colored solution (ignoring dark particles) were transferred from each tube to a well of a 96-well microtiter plate. The optical density was read at 600 nm with a multiwell spectrophotometer reader, setting blank to 0. The excitation wavelength of 600 nm is the optimal one, but wavelengths from 560 to 620 was considered. The percentage of fragmented DNA was calculated using the formula: % fragmented DNA = (S + T) / (S+T+B) x 100. Where S, T and B are the OD600 of fragmented DNA in the S, T and B fractions, respectively.

II.6. Intracellular ROS assay.

Intracellular ROS was measured using total ROS assay Kit 520 nm for

identifying ROS in cells by flow cytometry in the FITC channel. Briefly, the assay was done according to manufacture instructions as follows, a 500X stock solution of the ROS assay stain was prepared by adding 40 µL DMSO into the vial of ROS assay stain concentrate and mix well (stored at ≤-20°C, protected from light). The 500X ROS assay stain stock solution was used at 1X to label cells. It was added directly to cells in culture media at a final concentration of 1X by adding 2 µL of the 500X ROS assay stain stock solution for every one mL of cells; mix well. Alternatively, the 500X ROS Assay Stain stock solution was diluted to 1X using the ROS Assay Buffer. For each sample, 100 µL of ROS Assay Stain Solution was consumed. Then, cells were incubated for 60 minutes in a 37°C incubator with 5% CO_2, treated with the AgNPs to induce production of ROS. Analysis on a flow cytometer was conducted.

II.7. Statistical analysis:

All experiments were carried out in triplicate independent tests. Data was analyzed using SPSS (version 19) as mean and standard deviation (SD). Significant differences between treated and untreated cells were determined using Student's t test ; one-way analysis of variance (ANOVA). The results were considered statistically significant at probability ($P<0.05$).

ETHICAL APPROVAL: the study was approved by IRB - Faculty of Medicine Mansoura University; code R.21.08.1419.

III. RESULTS AND DISCUSSION

Characterization of AgNPs:

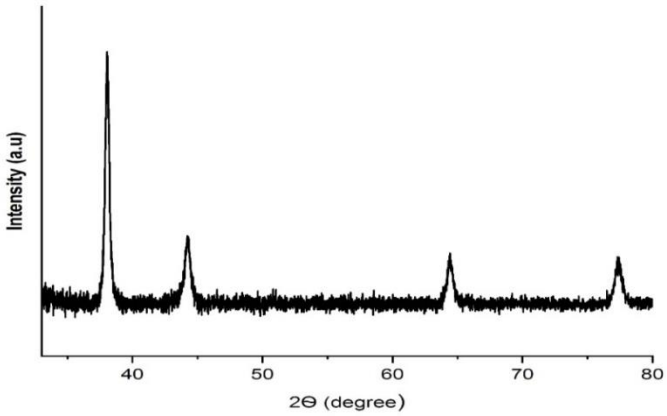

Figure (1): A typical XRD pattern of prepared AgNPs.

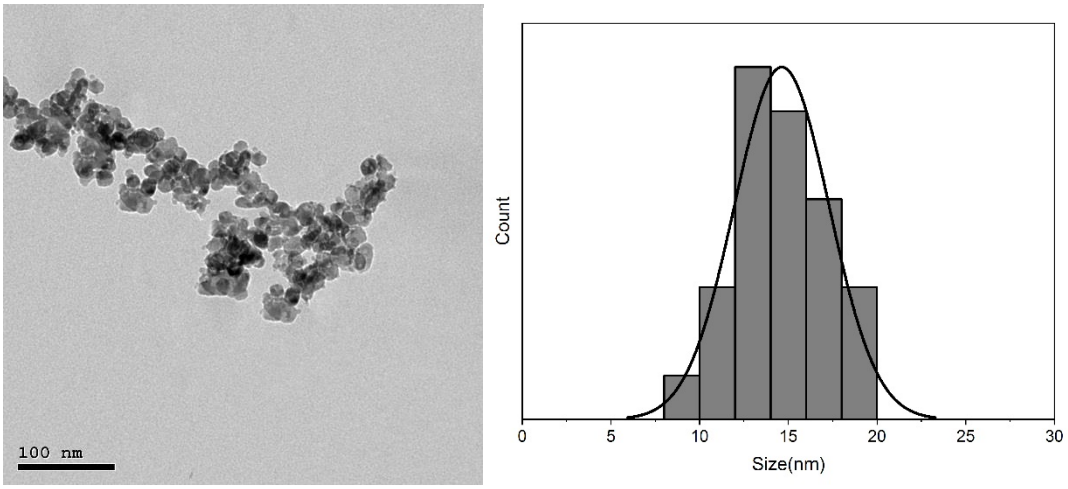

Figure (2): shows TEM image and particle distribution by nano measurement program

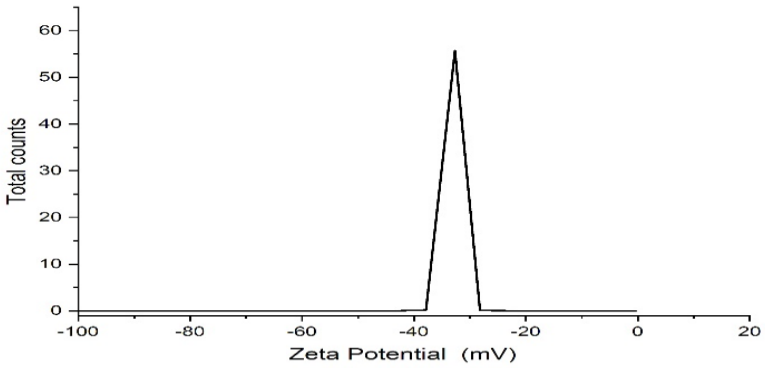

Figure (3): zeta potential pattern of prepared AgNPs

Figure (1) shows XRD curve of AgNPs best crystalline state without any additional peaks indicated the homogeny of chemical composition (no other peaks for the chemicals used in synthesis process). All peaks in the XRD pattern can be indexed as a face centered cubic (Fcc) structure (JCPDS, file no. 4- 0783). XRD pattern shows the presence of diffraction peaks corresponding to (111), (200), and (220) planes **(**Fatemeh et al., 2017). The size and morphology of silver nanoparticles were discuss using a TEM (Figure 2). TEM image shows silver nanoparticles with average diameter in range 10-20 nm and with spherical shape. Zeta potential result also determine the size of silver nanoparticles is about -32nm as shown in (Figure 3).

Cytotoxicity assessment:

Table (1): MTT assay using different concentrations of AgNPs treated HBF-4 cells.

Conc. (µg/mL)	Mean	SD	Viability %
0	0,573	0,011533	100
0,01	0,536333	0,016042	93,60093077
0,1	0,44	0,022271	76,78883072
1	0,261333	0,017388	45,60791158
10	0,15	0,036056	26,17801047
100	0,087667	0,004041	15,29959279

Conc.: conentrations**;** **µg**: microgram**;** **mL**: millimeter**;** **AgNPs:** silver nanoparticles; **SD**: standard Deviation

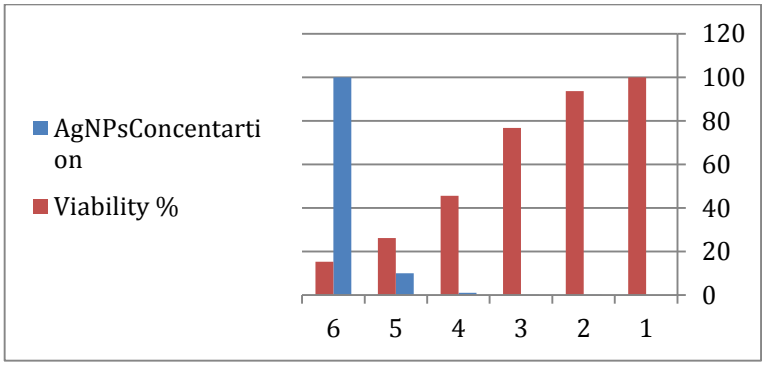

Figure (4): MTT assay using different concentrations of AgNPs treated HBF-4 cells.

The applications of AgNPs revealed a dose-dependent cytotoxicity on HBF-4 as shown in table (1) & Fig (4). LC_{50} was calculated to be 1.041 µg/mL.

DNA fragmentation assay and Intracellular ROS flowcytometeric assay:

HFB-4 cells were treated with two concentrations of AgNPs: the LC_{50} (1 µg/mL) and 1/10 of LC50 (0.1 µg/m)

Table (2): DNA fragmentation of AgNPs (at 0.1, 1 µg/mL) on HBF-4 cells.

Groups	DNA fragmentation%	SD	P1 Student's t test	P2 Student's t test	Test of significance
HFB-4 treated with 0.1 µg/mL	9.32	0.83	0.001*	< 0.001*	ANOVA F= 560.109 P < 0.001*
HFB-4 treated with1 µg/mL	27.32	1.21	< 0.001*		
Control group	4.63	0.38			

SD: standard deviation**; P1:** comparison of both study groups versus control group; **P2:** comparison between both study groups; *** p** is significant at < 0.05.

Table (3): Intracellular ROS assay of AgNPs (at 0.1, 1µg/mL) on HBF-4 cells.

Groups	ROS		P1 Student's t test	P2 Student's t test	Test of significance
	ΔRFU	% of control			
HFB-4 treated with0.1 µg/mL	140237.273	116.56	< 0.001*	< 0.001*	ANOVA F= 225.851 P < 0.001*
HFB-4 treated with 1 µg/mL	154017.462	127.63	< 0.001*		
Control group	120386.74	100			

S.D: standard deviation**; ROS:** reactive oxygen species**; P1:**comparison of both study groups versus control group; **P2:** comparison between both study groups**; RFU:** relative fluorescent unit; *** p** is significant at < 0.05.

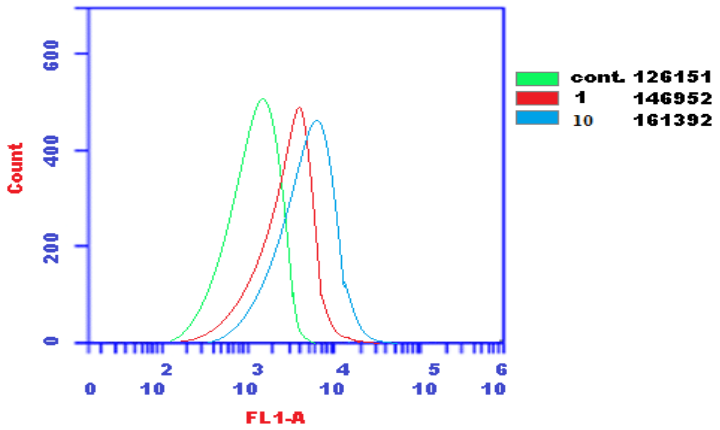

Figure (5): Intracellular ROS of HBF-4 cells exposed to various concentrations of AgNPs (0.1, 1 µg/mL) for 3 hours and intracellular ROS were determined using Flowcytometeric assay.

In the present study, table 2 shows significant increase in DNA damage in HBF-4 cells treated with IC_{50} and its 1/10 value in comparison to the control group. The level of damage significantly decreased by using 1/10 value of the LC_{50} compared to the higher dose, however, it is still significantly higher than the control group.

As well as, in table 3 and Figure 5, the release of IROS were significantly higher in both studied groups compared to their control group. Despite that the group treated with 1/10 value of the LC_{50} showed significant reduction compared to that group treated with the higher dose, it was still significantly higher than control group.

Silver nanoparticles are considered one of the most excessively used nanomaterial owing to their exponential broad-spectrum antibacterial properties. They have the highest degree of commercialization being as ingredients in 30% of nanoproducts (Chen et al, 2019). Recent advances and fast expanding applications of AgNPs has led to increased human exposure, which has raised concern regarding their safety risks (Gurunathan et al., 2018).

The toxicity of AgNPs has been demonstrated in a variety of in vivo and in vitro studies conducted on cancerous and non-cancerous cells. It was declared that AgNPs can modulate epigenetic dysregulation, which influence the cell cycle, and induce DNA hypermethylation. Their evident toxic impact has led to significant efforts in studying their cytotoxicity and genotoxicity (Franchi et al., 2015).

Human cell lines are sensitive tools for high-throughput toxicity screening that would reduce animal use for toxicological testing (Sahu et al., 2016); this can provide vital information in the field of cancer research. However, using in cancer cell lines in nanotoxicological studies, especially predictive toxicology studies remains questionable as multiple molecular explained pathways such as repair pathways are might be deregulated. This could be due to that cells which are lacking p53 or any tumor suppressor genes may be resistant to NP-induced cell death, and accumulate DNA damage which would influence all genotoxicity results (Franchi et al., 2015). Therefore, a comprehensive analysis for studying AgNPs toxicity effects especially those involving molecular mechanisms of the toxicity on normal, untransformed, human cells is a must. This study aimed to demonstrate the cytotoxic DNA

damaging effects and estimate the release of intracellular ROS of AgNPs on HBF-4 cells.

In the present study, there were observed dose-dependent reductions in the cell viability of AgNPs' treated cells. These results are in accordance to Lee et al. (2013) demonstrated that AgNPs treated NIH3T3 cells for 24 hr showed a dose-dependent reduction in cell viability with accumulation of AgNPs clusters and alterations in cell morphology within cytoplasm. They declared that AgNPs have been implicated in induction of cellular responses of cellular survival or death pathways. In 2015, Kaba and Egorova declared significant toxicity of AgNPs HeLa and U937 tumor cells using MTT assay, and cellular death pathway changes by flowcytometry.

Gurunathan et al. (2018) as well declared that AgNPs-induced loss of cell viability and cell death in NIH3T3 cells by increased oxidative stress and lipid peroxidation. Another study was conducted by Chen et al. (2019), they found that AgNPs induced decreased cell viability from 63% to 11% in a dose dependent manner in 2D keratinocytes. Similarly, Gao et al. (2017) found concentration dependent decrease of cell viability in adherent culture of differentiating mESCs treated with AgNPs at concentrations >1.0 μg/ml for 24 hours when measured by MTT assay. In 2018, Smith et al. declared that all sizes of silver nanoparticle caused 20% loss of cellular viability.

On the other hand, a previous study evaluated acute exposure of tissue cellular models to silver nanowires showed that there was no decrease in viability of human-reconstructed epidermis after exposure to with silver nanowires for 20 mins or 24 hrs (Lehmann et al., 2018). They explained that result in the epidermal model with the presence of stratum corneum and an extracellular matrix which may protect keratinocytes from cytotoxicity resulting from direct exposure to silver ions or AgNPs. As well, Sayed et al. (2019) declared that Ag NPs had negligible cytotoxic effects on HBF4 measured by MTT assay.

It has been demonstrated that one of the mechanisms by which AgNPs induced toxicity is disruption of plasma membrane leading to increased lactate dehydrogenase leakage and subsequently entering the cytoplasm with generation of ROS; a step toward induction of redox dependent cellular processes leading to apoptosis and DNA fragmentation. Similarly, AgNPs may induce their toxicity via affecting mitochondrial pathways by reducing

GSH and enhancing lipid peroxidation and ROS responsive genes expression, which cause DNA damage, and cellular death (Gurunathan et al., 2018).

In the present study, significant increase in release of IOR was observed on treated HBF-4 in comparison to the control group. This is in accordance with Gurunathan et al. (2018), they investigated the release of ROS after treating NIH3T3 cells with AgNPs using the 20,70-dichlorofluorescin diacetate (H2DCF-DA) assay; they found significantly increased ROS release compared with the control group after 24-h exposure. On the other hand, Chen et al. (2019) found no significant changes in intracellular ROS, MDA, or SOD in EpiKutis models after treatment with AgNPs compared to the significant increase resulted in treatment of 2D keratinocytes with equivalent dose of AgNPs.

According to previous studies, AgNPs oxidation in a complicated microenvironment like cells, usually occurs slowly, thus the balance of autonomous regulating mechanisms for this state of oxidative stress could remain for 24 hrs; after which chronic intracellular ROS accumulation usually overwhelm that cellular antioxidant defense mechanisms, resulting in increased oxidative stress over a period of 48 hrs (Chen et al., 2019).

Similarly, a significant increase in damage of DNA on treated HBF-4 cells was found in comparison to the control group. In agreement to these results, AgNPs displayed obvious oxidative DNA damage in a concentration dependent manner which resulted in apoptosis-mediated cell death (Franchi et al., 2015). As well, significant increase in DNA damage was observed in NIH3T3 Cells treated with AgNPs correlated to the significant increase in of 8-oxo-dG levels after exposure for 24 hr, this eventually caused genotoxicity in the form of DNA adducts and breaks, confirmed by using RNA-Seq analysis that suggested induction of epigenetics biological processes' alterations including nucleosome assembly and DNA methylation (Kaba and Egorova 2015; Gurunathan et al., 2018); they declared such effects were found in many different cell line studies such as human breast cancer cells, HT22 hippocampal neural cells, HeLa cells, A431 human skin carcinoma and A549 human lung carcinoma epithelial-like cells. These findings were explained by several in-vitro studies which demonstrated that AgNPs increased production of ROS, DNA damage, cell cycle arrest, leading

to cell death, besides, increase the upregulation of genes mostly p53, p21 and caspase-3 (Gao et al., 2017).

This study didn't support the results declared by Sayed et al. (2019) who stated that application of AgNPs on normal human melanocytes cells resulted in insignificant alterations in all assessed markers including Cytotoxicity assay, Caspase3, TNF-α and VEGFgenes levels' expression using q RT- PCR; in comparison to the control.

IV. CONCLUSION

Silver nanoparticles induced an increase in the intracellular ROS which indicate a state of oxidative stress, as well as it results in DNA damage. These results poses the carcinogenic potential of AgNPs in normal human melanocyte cells which may results in epigenetic changes on long term skin exposure. It is recommended to conduct further investigations for their genetic alterations mechanisms and to adjust the use of such chemicals in topical products manufacturing.

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الملخص العربى

السميـــة الجينيـــة لجسيمات فضـــة النـــانو على خط الخلايا الصبغية البشرية الطبيعية (خلايا HBF-٤)

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٢- قسم الفيزياء ، كلية العلوم ، جامعة المنصورة

٣- كلية العلوم ، جامعة القاهرة ؛ كلية النانو للدراسات العليا بالشيخ زايد

لقد إستخدم علم سموم النانو بسرعة في منتجات استهلاكية متنوعة ؛ التي أثارت مخاوف مختلفة. وتعد جسيمات فضة النانو هي واحدة من أكثر مواد النانو استخدامًا نظرًا لخصائصها الممتازة والواسعة المضادة للبكتيريا. ومع ذلك ، فإن الدراسات حول آثارها السامة على الخلايا والجينات نادرة. تهدف الدراسة الحالية إلى تقييم تأثير جسيمات فضة النانوعلى الحمض النووي وقياس مدى إطلاق أنواع الاكسوجين التفاعلية داخل الخلايا الصبغية البشرية الطبيعية (HBF-٤) .وقد أجريت الدراسة على الخلايا الصباغية البشرية الطبيعية (HBF-٤)، حيث تم معالجتها باستخدام جسيمات فضة النانو بجرعات مختلفة (٠,٠١ ، ٠,١ ، ١ ، ١٠ ، ١٠٠ ميكروغرام / مل) ، وتم تحديد جرعة ٥٠% من التركيز المميت باستخدام مقايسة الجدوى (MTT). ثم قسمت الخلايا إلى مجموعتين: الأولى عولجت بجرعة ٥٠% من التركيز المميت، والثانية عولجت بجرعة ٠,١ من جرعة ٥٠% للتركيز المميت. تم قياس تجزئة الحمض النووي باستخدام تقنية ثنائي فينيل أمين (DPA) وقياس أنواع الاكسجين التفاعلية داخل الخلايا ومقارنتها بمجموعة خلايا ضابطة. وقد لوحظ زيادات كبيرة ذات دلالات إحصائية في تلف الحمض النووي وإطلاق مختلف أنواع الاكسجين التفاعلية داخل خلايا HBF -٤ المعالجة بـجرعة ٥٠% للتركيز المميت وكذا المجموعة المعالجة بجرعة ٠,١ من جرعة ٥٠% للتركيز المميت مقارنة بالمجموعة الضابطة. الخلاصة: يمكن لجسيمات فضة النانو أن تسبب الإجهاد التأكسدي وتلف الحمض النووي في الخلايا الصبغية البشرية الطبيعية مع تأثير محتمل مسرطن. وهناك حاجة إلى مزيد من الابحاث لتشكيل آليات التعديلات الجينية الخاصة بهم.