Original Article

Impact of Silver Nanoparticles on the Ultraviolet Radiation Direct and Bystander Effects on TK6 Cell Line

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Abstract

Purpose/Aim: Ultraviolet C (UVC) radiation is harmful to cells and living organisms that cause direct and indirect DNA damage. UVC can also increase the inflammatory genes expression such as *COX-2* that results in elevated oxidative stress that plays a role in radiation-induced bystander effect (BSE). Silver nanoparticles (AgNPs) have used widely in commercial and medical products and the toxicological risks of AgNPs must be determined. The aim of this study was to investigate the direct and BSEs of UVC radiation and AgNPs on TK6 cell line. **Materials and Methods:** TK6 cells were exposed to AgNPs (10 µg/ml, 1 h). Then, they were exposed to UVC and to determine the BSEs of radiation, the irradiated cells media were transferred to nonirradiated cells. Expression level of *H2AX* and *COX-2* mRNAs were examined by quantitative real-time PCR and 8-OHdG formation was examined by ELISA. The cell viability examined by MTT assay. **Results:** $P < 0.05$ was considered as the level of significance. The results showed that the mean expression level of *H2AX* mRNA in the AgNPs + UVC group increased significantly in comparison with UVC group. 8-OHdG increased significantly in the BSE of UV group in comparison with sham control of BSE. *COX-2* mRNA increased significantly in the BSE of AgNPs + UVC with sham control in BSE. **Conclusions:** Our findings showed the induced DNA damage in TK6 cell by AgNPs and UVC radiation and also were seen BSE.

Keywords: Bystander effect, silver nanoparticles, TK6 cell, ultraviolet radiation

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Introduction

Ultraviolet radiation (UVR) is an electromagnetic radiation which is located between visible light and ionizing radiation. These radiations are divided into three categories according to their wavelength: ultraviolet A (315–400 nm), ultraviolet B (UVB) (280–315 nm), and ultraviolet C (UVC) (100–280 nm).[1] UVC radiation has the highest energy compared to other nonionizing radiations and is harmful to cells and living organisms, because the absorption spectrum of DNA, RNA, and protein is in this range.[2] A person can be exposed to UVC radiation by UV lamps which are without cover glasses, by electric arc welding with defective shields, and by sun radiation reaching earth's surface in areas that ozone layer has been damaged.[3] UVC radiation can cause oxidative DNA damage indirectly by generating reactive oxygen species (ROS) and thus producing 8-OHdG and single-stranded breaks.[4-8] High levels of 8-OHdG have been seen in different human and animal cancers.[9] In addition, UVC can express its genetic toxicity effects through DNA direct excitation, an enhancement in the production of cyclobutane pyrimidine dimers and (6–4) photoproducts which may cause primary and secondary DNA double-strand breaks. This type of mutation is seen in skin tumors. UVC can also produce GAMMA-H2AX. UVC can increase the inflammatory genes expression such as *COX-2*[4,10,11] that eventually results in elevated oxidative stress levels that play a role in radiation-induced bystander effect (BSE).[12,13] There is a direct correlation between *COX-2* upregulation and an increased risk of malignancy.[14-18]

The BSE of radiation is the inducing of biological changes in unexposed cells by signals transmitted from neighboring

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cells.[10] The ROS and reactive nitrogen species, as well as cytokines, interfere with the mechanism of BSEs.[19] Although this phenomenon is generally attributed to ionizing radiation, it also occurs in other stressors, such as UVR, chemotherapy drugs, and photodynamic therapy.[10] BSE includes a wide range of biological processes such as DNA damage, malignant transformation of chromosomal abnormalities, cell death, adaptive response,[19] premature and delayed mutations, and micronucleus formation.[20] Genes that play a role in inducing the BSE are often the same genes involved in the inflammation pathways. The most important of these genes are MAPKs, NFKB, iNOS, and *COX-2*.[12,13]

Silver nanoparticles (AgNPs) are the most abundant nanoparticles that are produced[4] and they are widely used in commercial products such as beauty products, deodorant, water treatment, and anti-sun lotion.[21] These nanoparticles have biomedical applications including the treatment of viral and fungi diseases, epilepsy, nicotine addiction and cancers, drug delivery, wound dressing, dental fillings, bone cement, contact lenses, and cardiovascular implants. Moreover, AgNPs can be used as light sensitizer or radiation sensitizer.[22-29] AgNPs can cause genotoxicity effects by increase of oxidative stress.[30,31] They can produce 8-OHdG, which is a biomarker for DNA damage caused by ROS. In addition, these nanoparticles can be attached directly to the RNA polymerase and prevent RNA transcription. They can also bind to DNA and change its structure.[32] Nanoparticles can produce bystander signals, for example, titanium dioxide nanoparticles induce high levels of oxidative stress, which leads to the expression of *COX-2*.[33] As mentioned *COX-2* have a contribution in the BSE.[12,13]

Therefore, due to the widespread use of AgNPs in medicine, it is necessary to know the mechanisms of their biological reactions, their potential for toxicity and to have profound knowledge of their impact on human health. Many studies have investigated the effect of AgNPs and UVR on human cells independently, but studies are very limited on the effect of AgNPs and UVR at the same time. In addition, no study has been done about the probability of BSE induction by AgNPs and UVR. This study investigated the direct and BSEs of AgNPs and UVC radiation on TK6 cell line using the MTT assay and genes expression of *COX-2* and *H2AX* as well as 8-OHdG biomarker quantity. The *H2AX* gene is a coding gene for DNA damage response proteins and is one of the oxidative DNA damage markers.[34-36] TK6 cells were exposed to UVC radiation after treatment with AgNPs (10 μg/ml) for 1 h. Then, to investigate the BSE, conditioned medium was prepared and unexposed cells were exposed to this conditioned medium, and then, the mentioned factors were measured in different groups.

Materials and Methods

Cell culture

TK6 human lymphoblastoid cells (ATCC No. CRL-8015) were initially cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum and 100 μl/ml antibiotic penicillin–streptomycin solution. The cells were then incubated at 37°C and 5% CO_2 incubator. Media were changed every 2–3 days until they reached 85% confluency.

Preparation of silver nanoparticles

AgNPs were purchased from the US Research Nanomaterial, Inc. (Stock #: US1038). For the preparation of AgNPs suspension, the nanoparticles were suspended in a deionized distilled water and immediately sonicated using the ultrasonic processor UP100H (100 watts, 30 kHz), (Hielscher, Germany). Final concentrations of nanoparticle suspensions were 10 μg/ml/cell culture wells.

Cells treatment with silver nanoparticles and ultraviolet C radiation

To perform MTT assay and ELISA and quantitative polymerase chain reaction (QPCR), TK6 cells in concentrations of 25×10^3 cells/well and 1.2×10^6 cells/well and 0.5×10^6 cells/well were used, respectively, (TK6 cells were seeded in plates, in the passage between 5 and 8). We divided the cells into different groups and treated as follow: UVC (1.1 mW/cm^2 irradiation for 20 min), AgNPs (10 μg/ml, 1 h), AgNPs + UVC (10 μg/ml for 1 h, and then 1.1 mW/cm^2 irradiation for 20 min), sham control in BSE (incubated cells with prepared conditioned medium from nonirradiated cells for 2 h), BSE of UVC (incubated cells with prepared conditioned medium from irradiated cells for 2 h), sham AgNPs in BSE (incubated cells with prepared conditioned medium from AgNPs treated cells), and BSE of AgNPs + UVC (incubated cells with prepared conditioned medium from treated with AgNPs and UVC irradiated cells). Thereafter, plates were incubated for 24 h at 37°C in a 5% CO_2 incubator. For each group, 3–6 separate wells were used.

After 24 h, the cells were treated with AgNPs at a final concentration of 10 μg/ml in each well and incubated for 1 h at 37°C in a CO_2 incubator. The plates were then removed from the incubator and half of the plates (the control and the bystander wells) were covered with autoclaved aluminum foil and the other half of the plates were exposed to UVC radiation (1.1 mw/cm^2) using germicidal UVC lamp (254 nm, 15 w) at a distance of 16 cm and for 20 min. Then, the cells were centrifuged with 1500 RPM for 6 min, and these samples were used to investigate the direct and the BSE of radiation.

Preparation of conditioned media to check the bystander effect

The cells were centrifuged immediately after exposure to UVC to prepare conditioned medium. Supernatant of the irradiated or AgNPs-treated cells was transferred to new tubes and recentrifuged to ensure that no cells were present in them. It was then ensured that no cell was present in the conditioned medium by optical microscope. Subsequently, the supernatant of the nonirradiated cells was discarded and the conditioned media were transferred to the tubes containing these cells. In other words, to examine the BSE, the conditioned medium was transferred to a tube containing their own bystander cells (sham

control in BSE, BSE of UVC, sham AgNPS in BSE, and BSE of AgNPs + UVC). Then, these treated cells with conditioned media were transferred to their own wells. Thereafter, the cells were incubated with this conditioned media at 37°C in CO_2 incubator for 2 h.

MTT assay

In this study, using MTT assay, the growth and cell viability of incubated TK6 cells with AgNPs and UVC radiation were investigated in direct and bystander groups. The steps of this test were: after the cells incubation with conditioned medium for 2 h, the plates were brought out from the incubator and 20 µl of MTT (5 mg/ml, Sigma Aldrich, M2128, USA) was added to each well and placed in an incubator for 4 h. Then, the plates were removed from the incubator and after centrifuge, cell supernatants were then drawn from the wells, and 150 µl of dimethyl sulfoxide (DMSO, Merck, USA) were added to each well in the dark and shaken for 45 min with 60 RPM. Eventually, the absorbances of each of the wells were read by an ELISA reader (State Fax, USA) at 540 nm. For each test, 4–8 separate wells were considered.

RNA extraction and quantitative polymerase chain reaction to verify the expression of H2AX and COX-2 mRNA

As mentioned above cell culture and treatment of cells with AgNPs and UVC radiation were performed. In the next step, cells supernatant of all samples that were centrifuged, discarded, and the total RNA were extracted using a RNA extraction kit (Cinaclon CO, RNX-Plus Cat NO.EX6101). Quality and concentration of RNA were determined using Nano Drop (ND 1000, Thermo, USA). To remove possible contamination of the RNA sample with DNA was used the DNase I, RNase-free kit (Thermo, Cat. No. EN0525). Then, the cDNA synthesis was performed using the Japanese TAKARA kit (Cat. No. RR037A) and using 1 µg of RNA of each sample, by PCR System (Astec, BL-516H japan). After that, the prepared cDNA was stored in a −20°C freezer. To determine the amount of intended mRNA in the samples, the cDNA was amplified using Real-time PCR and SYBR Green i PCR Mix Amplicon Kit (Cat. No. A325402, Denmark). The β-actin gene was used as an internal control gene. The Q-PCR reaction was performed using the Real-Time PCR 7500 (Applied Biosystem, USA). The used primers in this study and QPCR program are listed in Tables 1 and 2.

Measurement of 8-OHdG biomarker by ELISA method

To measure the 8-OHdG biomarker in the samples, ZellBio GmbH Kit (Cat. No: ZB-1436-H9648, Germany) was used and samples were prepared according to the protocol of the kit: after treating the cells with AgNPs and radiation, as mentioned above, cells were centrifuged. The supernatant of the cells was discarded and 2.5 ml of sterilized phosphate-buffered saline (PBS) buffer (PBS tablet in 500 ml of deionized water, Gibco, USA) was added to the Falcon tube, and again, the cells were centrifuged. Then, the cells supernatant was discarded and

Table 1: The sequences of the COX-2, H2AX, and ACTB primers for quantitative polymerase chain reaction analysis

Primer name	Sequence (5'-3')	Length (bp)
COX2-F	AGG GTT GCT GGT GGT AGG AA	64
COX2-R	GGT CAA TGG AAG CCT GTG ATA CT	
H2AX-F	CAA CAA GAA GAC GCG AAT CA	223
H2AX-R	CGG GCC CTC TTA GTA CTC CT	
ACTB-F	GGG AAA TCG TGC GTG ACA TTA AGG	183
ACTB-R	GGA AGG AAG GCT GGA AGA GTG C	

Table 2: The quantitative polymerase chain reaction program

Step	Number of cycle	Temperature (°C)	Time
Denaturation	1	95	2 min
Denaturation	45	95	30 s
Annealing		60	40 s
Extension		72	30 s
Final extension	1	72	5 min

PBS was added to the cells in a way that the cell concentration was 2×10^6 cells/ml. After then, the cells were transferred into 2-ml microtubes and stored in a −70°C freezer. Then, on the day of the experiment, the microtubes were brought out of the freezer and were kept at 4°C and after melting the contents of each of the microtubes were sonicated until the cells were lysed. After sonication of the samples, the microtubes were centrifuged at 12,000 RPM for 10 min. Cells supernatant was then removed carefully and transferred to other microtubes and stored at −80°C. The supernatant was used to measure the 8-OHdG. The 8-OHdG antibody was embedded in the ZellBio kit. After adding cell supernatant into these wells, 8-OHdG of samples was attached to this antibody. Then, the second antibody which is attached to the biotin binded to 8-OHdG. Later, streptavidin-HRP was added, which had a high affinity for biotin. After washing with a buffer, the chromogens were added to the wells. Eventually, after stopping the reaction by the stopping solution, the amount of light absorption was read by ELISA reader.

Statistical analysis

Prism software (version 6.07, ©1992-2015 GraphPad Software, Inc) was used to analyze the data. The results were analyzed using ANOVA and the *post hoc* Tukey test. $P < 0.05$ was considered as the level of significance.

Results

The effect of silver nanoparticles and ultraviolet C radiation on the cell viability

Statistical analysis showed that there was no significant difference between the average cell viability in UVC, AgNPs, and AgNPs + UVC groups in comparison with the control group ($P > 0.05$) [Figure 1].

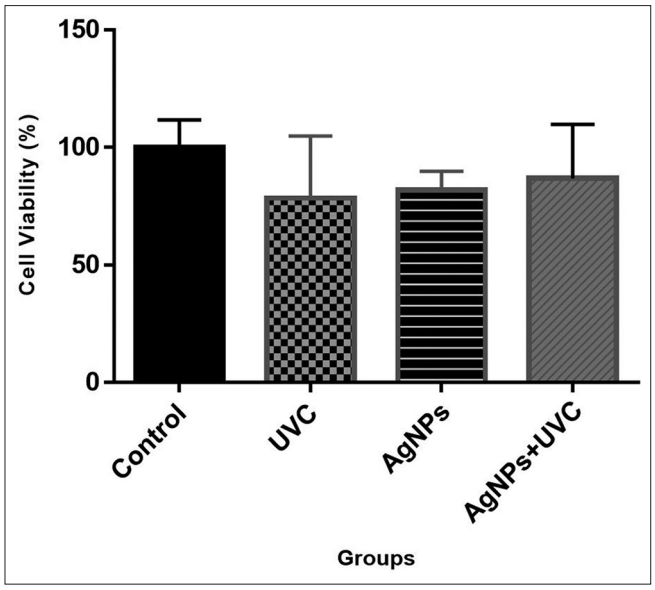

Figure 1: Comparison of cell viability (%) in control, ultraviolet C, silver nanoparticles, and silver nanoparticles + ultraviolet C groups. The results are displayed as mean ± standard deviation from the results of the experiment in 6–8 separate wells for each group

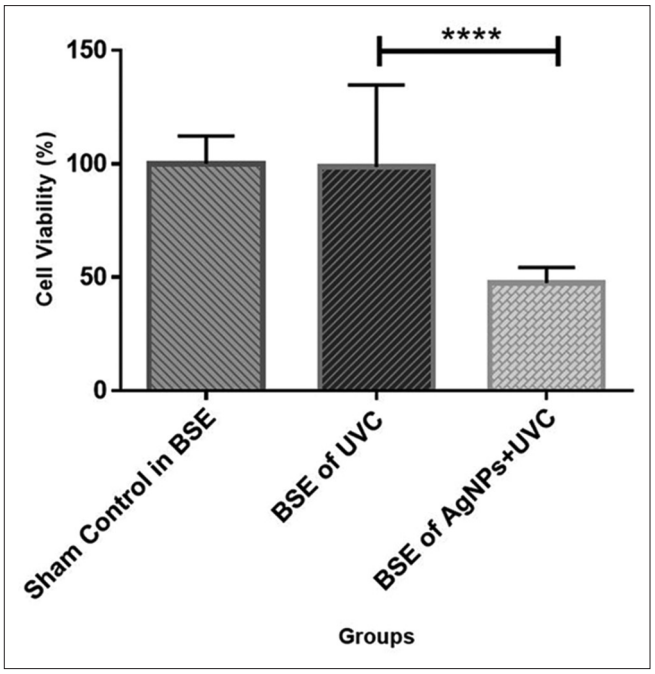

Figure 2: Comparison of cell viability (%) in sham control in bystander effect, bystander effect of ultraviolet C, and bystander effect of silver nanoparticles + ultraviolet C groups. The results are displayed as mean ± standard deviation from the results of the experiment in 6–8 separate wells for each group. The numbers of stars show the level of significance. (****: $P < 0.0001$)

The effect of silver nanoparticles and ultraviolet C radiation on the cell viability of bystander groups cells

Statistical analysis showed that the average cell viability decreased in BSE of UVC and BSE of AgNPs + UVC groups in comparison with sham control in BSE but was not statistically significant. Cell viability in BSE of AgNPs + UVC was significantly lower than BSE of UVC group ($P < 0.0001$) [Figure 2].

The effect of silver nanoparticles and ultraviolet C radiation on COX-2, H2AX mRNA expression levels and 8-OHdG formation using the ELISA method in TK6 cells

Comparison of H2AX mRNA levels

Statistical analysis showed that the average level of *H2AX* mRNA expression in the UVC and AgNPs and AgNPs + UVC groups was significantly higher than the control group ($P < 0.0001$). Furthermore, the average level of *H2AX* mRNA expression was significantly higher in the AgNPs + UVC group than the UVC group ($P < 0.001$) [Figure 3].

Comparison of COX-2 mRNA expression

Statistical analysis showed that the average level of relative expression of *COX-2* mRNA was significantly higher in UVC, AgNPs, and AgNPs + UVC groups than the aforementioned level control group ($P < 0.0001$). The mean relative expression level of *COX-2* mRNA in the AgNPs + UVC group was statistically significantly lower than that of the UVC group ($P < 0.001$) [Figure 4].

Comparison of 8-OHdG formation amount

Statistical analysis showed that the mean value of the 8-OHdG increased slightly in the UVC and AgNPs + UVC group in comparison with the control group ($P > 0.05$). However, the formation of the 8-OHdG decreased significantly ($P < 0.0001$) in the AgNPs group in comparison with the control group [Figure 5].

The effect of silver nanoparticles and ultraviolet C radiation on the caused bystander effect on TK6 cells by examining the expression of COX-2, H2AX mRNA, and measurement of 8-OHdG value by ELISA method

Comparison of H2AX mRNA expression in bystander groups

Statistical analysis showed that the expression of *H2AX* mRNA increase was not significant in BSE of UVC and sham AgNPs in BSE ($P > 0.05$) in comparison with sham control in BSE. In addition, the expression level of *H2AX* mRNA decreased in the BSE of AgNPs + UVC in comparison with sham control in BSE, BSE of UVC, and sham AgNPs in BSE, but this decrease was not statistically significant ($P > 0.05$) [Figure 6].

Comparison of COX-2 mRNA expression in bystander groups

Statistical analysis showed that the mean relative expression level of *COX-2* mRNA in BSE of UVC and sham AgNPs in BSE increased in comparison with the sham control in BSE, but it was not significant ($P > 0.05$). Moreover, the relative expression level of *COX-2* mRNA increased in the BSE of AgNPs + UVC compared to sham control in BSE, BSE of UVC, and sham AgNPs in BSE, and this increase was statistically significant in BSE of

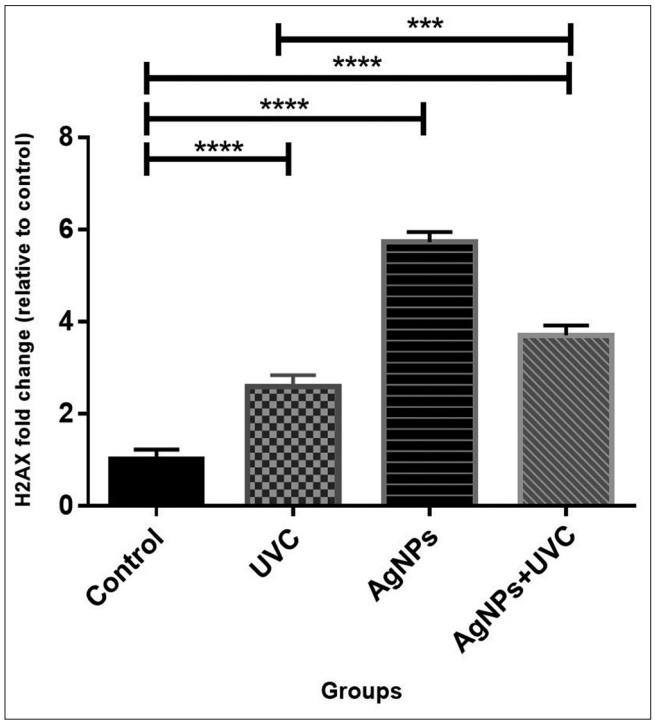

Figure 3: Comparison of the H2AX gene expression in ultraviolet C, silver nanoparticles, silver nanoparticles + ultraviolet C, and control group. The results are displayed as mean ± standard deviation from the results of the experiment in three separate wells for each group. The numbers of stars show the level of significance. (***: $P < 0.001$), (****: $P < 0.0001$)

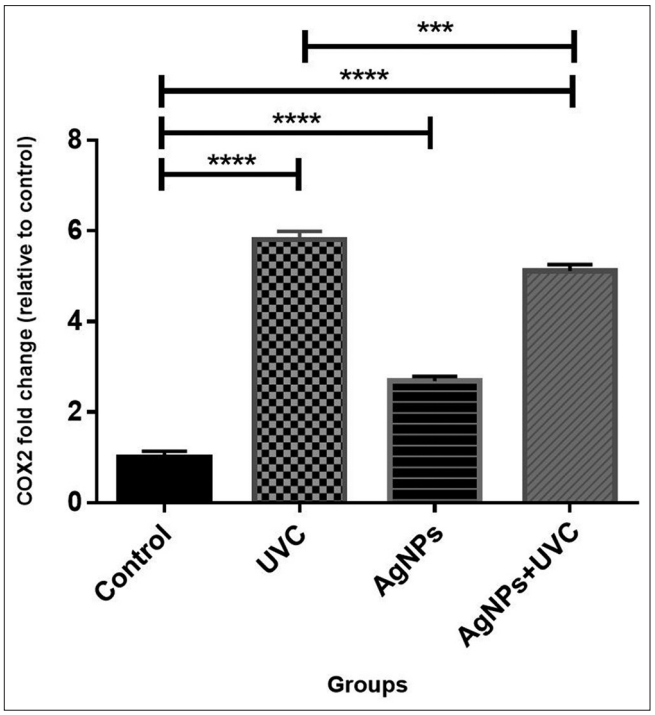

Figure 4: Comparison of the COX-2 gene expression in ultraviolet C, silver nanoparticles, silver nanoparticles + ultraviolet C, and control group. The results are displayed as mean ± standard deviation from the results of the experiment in three separate wells for each group. The numbers of stars show the level of significance. (***: $P < 0.001$), (****: $P < 0.0001$)

AgNPs + UVC in comparison with the sham control in BSE ($P < 0.05$) [Figure 7].

Comparison of 8-OHdG in bystander groups

Statistical analysis showed that the mean value of the 8-OHdG in the BSE of AgNPs + UVC groups was not significant in comparison with the sham control in BSE ($P > 0.05$). However, the mean value of 8-OHdG in the BSE of UVC and sham AgNPs in BSE was significantly higher than sham control in BSE ($P < 0.0001$). The mean value of the 8-OHdG in the BSE of AgNPs + UVC was significantly lower than the BSE of UVC and sham AgNPs in BSE ($P < 0.0001$) [Figure 8].

Discussion

In our study, treatment with AgNPs before UVC exposure to cells did not cause a significant difference in cell viability in comparison with the control group. This result was confirmed by the results of the Zare *et al.* study.[37] Our study contrasted with the study of Espinha *et al*.[38] In that study, UVC radiation caused a significant decrease in cells viability and the used UVC radiation was much higher than that in our study.

In the bystander groups of this study, the conditioned medium of the irradiated cells (BSE of UVC) did not result in a significant difference in the cell viability in comparison with the sham control in BSE, which confirmed the results of the Ghosh *et al.* and Banerjee *et al.* studies.[39,40] The conditioned medium prepared from cells that were pretreated with AgNPs and then were irradiated with UVC radiation (BSE of AgNPs + UVC) did not cause a significant difference in cell viability in comparison with the sham control in BSE but showed a significant decrease in comparison with the BSE of UVC, which can show the synergistic effect of AgNPs and UVC radiation in bystander cells.

The results of our study showed that the mean level of relative expression of *H2AX* gene increased significantly in the UVC, AgNPs, and AgNPs + UVC groups compared to the control group, which confirmed the results of the Zare *et al.* study.[37] Moreover, a significant increase in the mean level of relative expression of *H2AX* gene in the AgNPs + UVC group compared to the UVC group showed the synergistic effect of UVC and AgNPs in damaging the DNA and thereby increasing the expression of the *H2AX* gene. In bystander groups, the expression of *H2AX* gene was not significant in the BSE of UVC and sham AgNPs in BSE and BSE of AgNPs + UVC compared to the sham control in BSE.

In this study, the *COX-2* gene expression increased significantly in UVC, AgNPs, and AgNPs + UVC groups in comparison with the control group, which is indicative of caused oxidative stress in groups. In the bystander group, the expression of this gene increased significantly in the BSE of AgNPs + UVC compared to the sham control in BSE. László *et al.* investigated the level of *COX-2* expression in MEFS/S cells (wild-type mouse

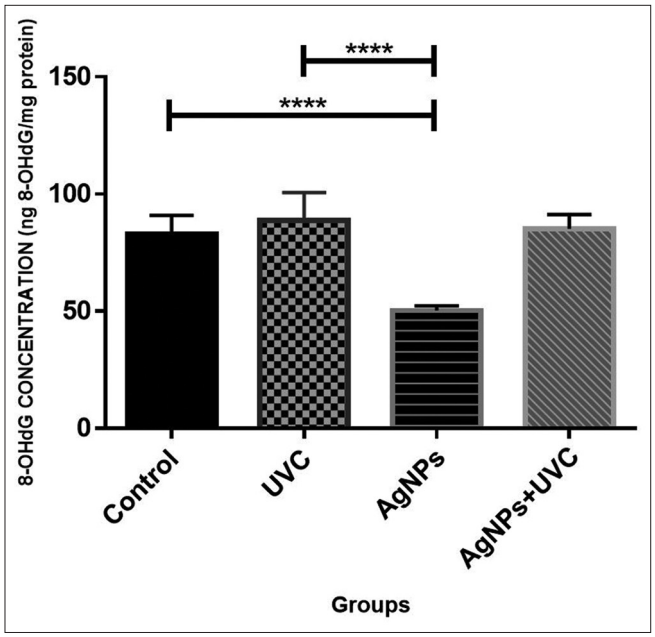

Figure 5: Comparison of the mean value of 8-OHdG in control, ultraviolet C, silver nanoparticles, and silver nanoparticles + ultraviolet C groups. The results are displayed as mean ± standard deviation from the results of the experiment in three separate wells for each group. The numbers of stars show the level of significance. (****: $P < 0.0001$)

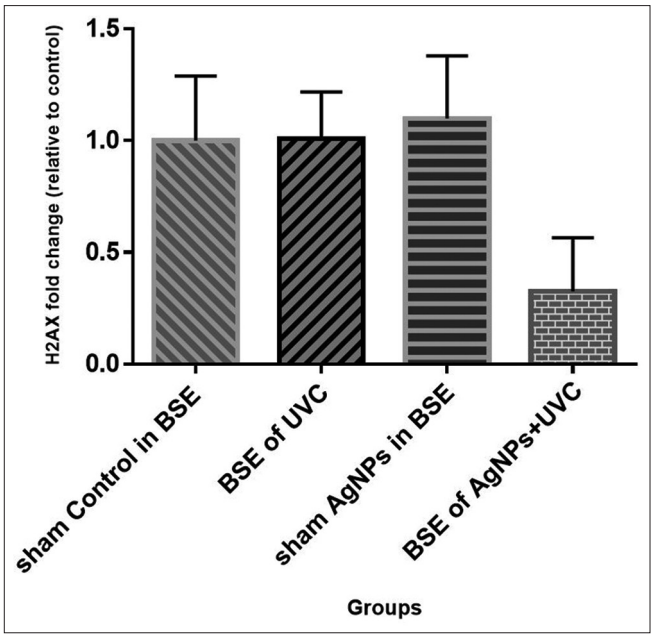

Figure 6: Comparison of the *H2AX* gene expression in sham control in bystander effect, bystander effect of ultraviolet C, sham silver nanoparticles in bystander effect, and bystander effect of silver nanoparticles + ultraviolet C groups. The results are displayed as mean ± standard deviation from the results of the experiment in three separate wells for each group

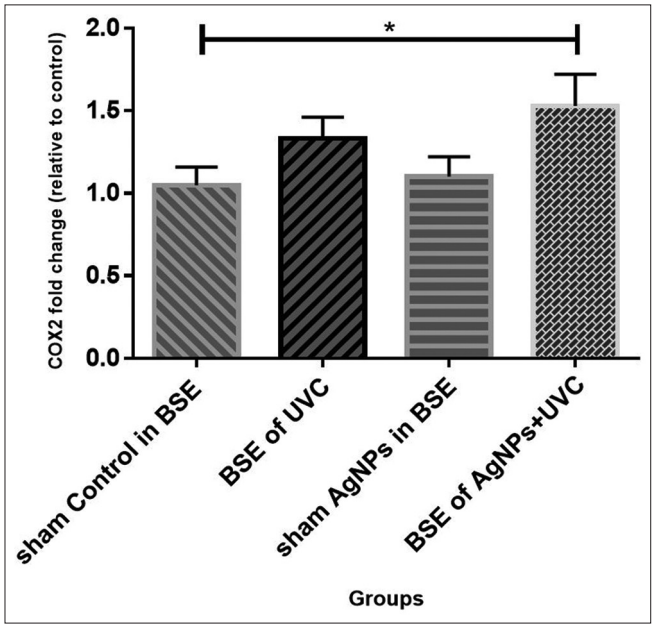

Figure 7: Comparison of the COX-2 gene expression in sham control in bystander effect, bystander effect of ultraviolet C, sham silver nanopar ticles in bystander effect, and bystander effect of silver nanoparticles + ultraviolet C groups. The results are displayed as mean ± standard deviation from the results of the experiment in three separate wells for each group. The numbers of stars show the level of significance. (*: $P < 0.05$)

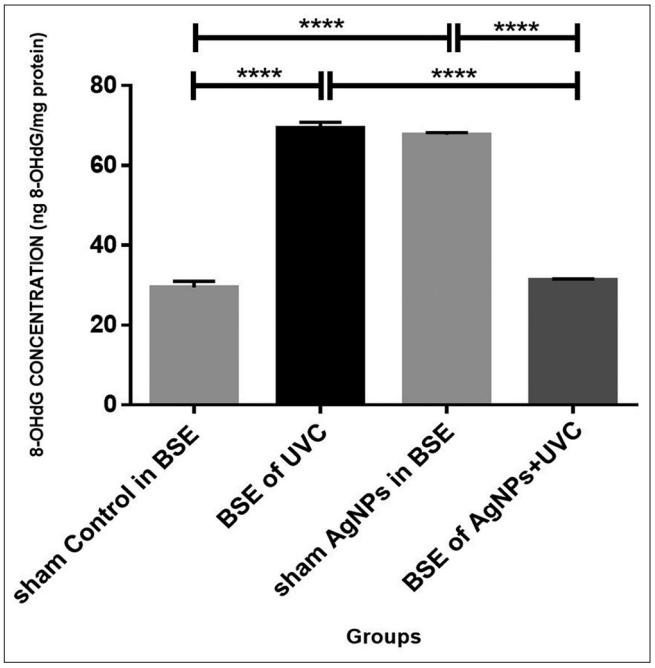

Figure 8: Comparison of value of 8-OHdG in sham control in bystander effect, bystander effect of ultraviolet C, sham silver nanoparticles in bystander effect, and bystander effect of silver nanoparticles + ultraviolet C groups. The results are displayed as mean ± standard deviation from the results of the experiment in three separate wells for each group. The numbers of stars show the level of significance. (****: $P < 0.0001$)

embryo fibroblasts) exposed to 30 J/m^2 UVC and observed that *COX-2* gene expression increased after UVC radiation and their results were in line with our study.[41]

In our study, the amount of 8-OHdG decreased significantly in the AgNPs group compared to the control group and UVC.

The amount of this factor in the BSE of AgNPs + UVC was not significantly different from sham control in BSE and significantly decreased compared to the BSE of UVC, while 8-OHdG increased significantly ($P < 0.0001$) in the BSE of UVC and Sham AgNPs in BSE in comparison with the sham AgNPs in BSE.

Yin *et al*. examined the effect of UVB radiation of 240 and 480 J/cm^2 on 8-OHdG formation in the JB6 cell line. UVB radiation resulted in a significant increase in the formation of 8-OHdG in cells, which contradicted our results. The used radiation energy in our study was much less than that study and that probably caused the differences in the results of two studies.[42] Zhao *et al.* used AgNPs, in the concentration range of 0–1 mg/ml for the treatment of MCF-7 cells, and the formation of 8-OHdG increased significantly in these cells ($P < 0.01$) in comparison with the control group. However, our study showed a significant decrease, probably due to the low concentration of used AgNPs in our study.[32] Tyagi *et al.* investigated the formation of 8-OHdG in HaCaT cells treated with AgNPs at a concentration of 2 μg/ml for 3 h. The results of this study showed that pretreatment of cells with AgNPs and then UV radiation caused a decrease in the amount of 8-OHdG in comparison with the UV radiation group, which contradicted the results of our study and showed the protective role of AgNPs in this concentration (2 μg/ml) against UV radiation. Moreover, in that study, the pretreatment of cells with AgNPs and then UV radiation reduced the amount of 8-OHdG in comparison with the control group, which contradicted the results of the present study.[43]

Conclusions

In general, the genetic toxicity effect of cell treatment with AgNPs and UVC radiation separately and together in comparison with the control group in TK6 cells was demonstrated by increasing the expression of *H2AX* and *COX-2* genes. In addition, treatment of cells with AgNPs and then UVC radiation increased the expression of *H2AX* gene in comparison to the single UVC radiation group. However, no significant differences were observed in the amount of 8-OHdG between the treated cells with AgNPs and irradiated (AgNPs + UVC) in comparison with the control group. In the bystander groups, treated cells with AgNPs and UVC radiation decreased the formation of 8-OHdG, which showed the transmission of the BSE signals. The *COX-2* gene expression was increased in the bystander groups treated with AgNPs and UVC radiation in comparison to the sham control in BSE group which is indicative of the bystander signal transmission.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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