Research Article
Volume 12:3, 2021

Journal of
Cytology & Histology

ISSN: 2157-7099 Open Access

Histopathological, Biochemical and Genetic Profile Post Application of Free Silver Nanoparticles and H_2O_2 Combined Form in Vitro Study

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Abstract

Current studyaimed to evaluate the antiviral activities of free silver nanoparticles (AgNPs) and hydrogen peroxide combined silver nanoparticles(AgNP-H2O2) compared to standard IFN-α2a against Herpes simplex virus type-1 (HSV-1), Vesicular stomatitis virus (VSV) and Middle East respiratory syndrome-coronavirus (MERS-CoV). The related biochemical and pathological changes as well asthe expression levels of antiviral biomarkers, namely Mx-A and 2'-5'-oligoadenylate synthetase (2'-5'-OAS)were investigated. Cytotoxicity of AgNPs and AgNP-H2O2 was monitored against Vero cellsusing MTT assay. Data revealed that the toxicity was concentration dependent and AgNP-H2O2was significantly ($P<0.05$)toxicthan AgNPs recording an IC_{50}value of 0.864 and7μM/ml, respectively. Oxidative stress markers showed elevated reactive oxygen species and decreased lactate dehydrogenase post treatment with AgNPs and AgNP-H2O2 compared to untreated cellcontrol. Assessment of the antiviral activity of tested particles prior to infection showed thatAgNP-H2O2exhibited higher reduction in the viralinfectivity titer in the order of 76.21% and 57.89% compared to that recorded post infection (34.11% and 36.84%) against HSV-1 and VSV, respectively. Similar pattern was observed in case of AgNPs but with lower depletion rate while, the highest depletion was in case of IFN-α2a. The percentage reduction in the plaque forming units against MERS-CoV were 33.3%, 66.6% and 87.5% post treatment with AgNPs, AgNP-H2O2 and IFN-α2a, respectively.MxA and2'-5'-OAS genes exhibited a signifinat ($P<0.05$) up-regrulationin. Pathological changes post treatment with tested particles showed signs of apoptosis indicating that although these particles exhibited promising antiviralpotentials, buttheir toxicity profile should be considered.

Keywords: Silver Nanoparticles • Hydrogen peroxide • Fetal bovine serum • Vesicular stomatitis virus • MERS-CoV

Introduction

Silver nanoparticles (AgNPs) are well-recobnized antimicrobial agents effective against a wide range of bacteria and fungi [1-4]. Antibacterial and antifungal activities of AgNPs are primarily due to the inhibitory effectofthe released silver ions on respiratory enzymes [1,5]. The antiviral activities of AgNPs against numerous viruses such as HIV-1 [6,7], hepatitis B [8], herpes simplex [9], respiratory syncytial [10], monkeypox [11], tacaribe [12], and H1N1 influenza A virus [13,14] have beenalso reported. Unlike its antibacterial and antifungal activities, the major antiviral mechanism of AgNPs is likely due to the physical inhibition of binding between the virus and the host cell. A correlation between the size of AgNPsand their antiviral activities was observed for some viruses such as HIV-1, whereit was found that AgNPs smaller than 10 nm specifically inhibited HIV-1 infection [6]. Despite the promising antimicrobial properties of silver based nanoparticles, there are some concerns regarding the biological and environmental risks of utilizing these particles. It wasdemonstrated that AgNPs had adverse effects, such as cytotoxicity and genotoxicity on aquatic organisms like fish [15] and could inhibit photosynthesis in algae [16]. A study carried out on mammals showed a significant decline in mouse spermatogonial stem cells following the administration of AgNPs [17]. Therefore, preventing the diffusion and intake of AgNPs into the environment and the biosphere are important considerations in the design of silver based antimicrobial nanoparticles [18-21]. One of theseapproachesis the fixation of AgNPs into matrices; for example, AgNPs-coated polyurethane demonstrated antiviral activity against HIV-1 and herpes simplex virus [22]. H_2O_2 is also a convenient means for virus inactivation,it was reported that 3% H_2O_2 could inactivate adenovirus types 3, 6, and 4, rhinoviruses 1A, 1B, and 7, myxoviruses, influenza A and B, respiratory syncytial virus, and coronavirus 229E strain within 1-30 mins. Corona and influenza viruses were found to be the most sensitive, whereas Reoviruses, adenoviruses and adeno associated virus were relatively stable [23]. Recently, it was also reported that H_2O_2 could inactivate rabies virus in a time dependent manner till reaching complete inactivation within 3 hrs resulting in the production of a potent immunogenic rabies vaccine [24]. Consequently, the aim of the present work is to evaluate the antiviral potentials of both freesilver nanoparticles as well as silver nanoparticles combined hydrogen peroxide against RNA and DNA viral models. Theassociatedoxidative stress profile, pathological

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Received 24 September 2020; **Accepted** 18 March 2021; **Published** 25 March 2021

changes in addition to the transcriptomic analysis of antiviral genetic markers were also investigated.

Materials and Methods

Cell culture

African Green Monkey kidney (Vero) cells (CCL-81) were kindly supplied from the International Center for Training and Advanced Researches(ICTAR-Egypt), at Passage No.142. Cells were maintained according to the producer manufacturing protocol. The cells (1x10^5/ml) were cultured in Dulbecco's modified Eagle's medium E-MEM(Hyclone-USA), supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM/ml Glutamine, which were all purchased from Sigma Aldrich, USA. Cells were incubated in a humidified atmosphere of 5% CO_2 at 37˚C(Jouan-France).

Silver Nano-particles and Interferon-α2a

AgNPs and AgNP-H_2O_2 were kindly supplied from pharmacology department, faculty of pharmacy, Al-Azhar university. Interferon-α2a (INF-α2a) waskindly obtained fromThe National Organization for Drug Control and Research (NODCAR).Silver nanoparticlesand INF- α2a were in a final concentration of 1mM and 0.5 mg/dose; repectively.

Virus models

Herpes simplex virus type-1 (HSV-1) as DNA virus model andVesicular stomatitis virus (VSV) Indiana strain-156 as RNA virus model, were kindly supplied from The International Center for Training and Advanced Researches (ICTAR-Egypt). MERS-CoV(RNA virus model) was kindly providedby Prof.Dr. Mohamed Ahmed Ali, prof. of virology and molecular genetics, The Center of Excellence for influenza virus, National Research Center, Giza. Viruses were propagated according to the procedure previously described by Ramadan, *etal*,(2009) [25].

Cell viability assay

Cytotoxic effect of differnet concentrations of AgNPsandAgNP-H2O2 was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diph enyl-tetrazolium bromide (MTT) assay, where growth medium was decanted from pre-cultured Vero cells 96-well micro titer plates. AgNPsandAgNP-H_2O_2 were applied ata starting concentration of 100µM/ml in a series of double fold serial dilution in serum free E-MEM as 0.1 ml of each dilution/well. Negative untreated cell control was considered and plates were incubated at 37ºCfor 24 hrs. Post incubation.Plates were washed three times with phosphate buffer saline (PBS) as 250µl/well. Fifty µl of MTT solution (0.5 mg/ml) were added as 50µl/well and plates were incubated for a further 4 hrs at 37ºC. Plates were PBS washed as previousand the formed purblecolored formazan crystals were dissolved using 50 µl/wellof DMSO (Sigma Aldrich-USA).Plates were shaked for 5 mins at room temperature. Optical density (OD) was measured at 570 nm using an ELISA plate reader. The percentage of cellular viability was calculated using the following formula:

Viability % = Mean OD of test wells X 100

Mean OD of control wells

Also, the half maximal inhibitory concentration (IC_{50}) value was determined as the concentration resulting in 50% cell growth inhibition following 24 hrs exposure to AgNPs andAgNP-H2O2 compared to the untreated control cells using Masrplex-2010 software [26].

Biochemical analysis

Assessment of Lactate dehydrogenase B (LDH-B) and reactive oxygen species (ROS) levels were determined following Vero cell treatment with the IC_{50} of AgNPs and AgNP-H_2O_2according to the manufacturer's protocol using ELISA kits biovision, Catalog No.ab183367 and K936-100, respectively.

Antiviral activity

Ten fold serial dilutions of virus models namely Herpes simplex virus type-1 (HSV-1), Vesicular stomatitis virus (VSV) were dispensed as 100 µl/well in96-well plates precultured Vero cells post decanting theirgrowth media. Each virus dilution was added to 8 wells and plates were incubated at 37ºC to allow virus adsorbtion. Plates were incubated for 7 days with daily microscopic examination(Hund-Germany) for the detection of cytopathic effect (CPE). Viral infectivity titer was evaluated by determining fifty-percent tissue culture infective dose ($TCID_{50}$) according to the method adopted by Reed and Muench, (1938)[27].

Direct antiviral activity was performed according to the method described by Sala *et al*., (2018), where 10-foldserially diluted HSV-1 and VSV models were applied for one hr at 37ºC post decanting the growth media on Vero cells pre-cultured plates. Infection medium was decanted from the plates and the infected cells were PBS washed three times to remove unadsorbed viruses. IFN-α2a (10 ng/ml) as well as safe concentrations of AgNPs (2.75 µM/ml) and AgNP-H_2O_2 (0.4 µM/ml) were applied to the previously infected plates. Plates were incubated for 24 hrs at 37ºC and microscopically examined for detection of CPE [28].

Indirect antiviral activity was performed by cell treatment with the safe concentrations of safe concentration of test nanoparticles formulae for 24 hrs at 37ºC. Treatment medium was decantedfollowed by Vero cell infection with 10-fold serially diluted viruses [29]. Percentage reduction of the virus

infectivity titer using both methods was determined as previously described in treated plates compared to untreated plates.

Plaque assay

AgNPs and AgNP-H_2O_2 and IFN-α2a were examined for their inhibitory effect on Middle East respiratory syndrome-coronavirus (MERS-CoV) according toBaer *et al.,* (2014). Ten fold serially MERS-CoVwas inoculated into12-well Vero cells precultured plates for 1 hat 37°C to allow adsorption.The cells were PBS washed to remove the non-adsorbed virus.Plaque assaywas performed by pouringsterile heated (45°C) 1:1 mixture of 0.6% agaroseand 2X medium containing 10% FBSin all plates. Plates were left at room temperature for solidification followed by incubation to allow the formation of clearly countable plaques. Infected plates were daily examinedfor detection of plaques. Agaroseoveralyer was removed post 18hrscellular fixation using 10% formaline-saline mix. Fixed cells were then stainedusing1% crystal violet for 15 mins followed by washing with water.Plaques were counted in treated and untreated plates and the percentage reduction was calculated [30].

MxA and 2'-5'-OAS gene expression levels

Expression levels of MxA and2'-5'-oligoadenylate synthetase (2'-5'-OAS) genes were evaluated using real time PCR, where total RNA was extracted from Vero cells treated with IC_{50} ofAgNPs and AgNP-H2O2as well as untreatedcellsusing Gene JET RNA Purification kit (Fermantus-UK) according to the manufacturer's instructions. The concentration and the purity of the extracted RNAwere determinedby measuring the absorbanceat 260 and 280 nm. Extracted RNA (1 µg) was reverse transcripted to cDNA using Quantitect Reverse Transcription kit (Qiagen-Germany).The expression levels of MxAand 2'-5'-OAS geneswereestimatedusing the following primers,Mx-A [F 5'-AAA TGG CTC AAG AGG TGGA-3' R 5'-TAT CGC TGA CAG TTG GGTG-3'] and 2'-5'-OAS [F 5'-TGA CGG TCT ATG CTT GGG AG and R 5' -CAA GAT GCA CTG GCA TTC AG-3']. Melting curves were applied to demonstrate the amplification of therequired product. In the mean time, a standard curve was performed to evaluate the amplification efficiency and the relative fold changes regarding the expression levels.

Pathological changes

The effect of the tested particles on the cellular level was determined via detection of pathological changes developed post Vero cell treatment with the IC_{50} of AgNPs and AgNP-H_2O_2 using hematoxylin and eosin stainingvaccording to [31]. Fifty microliters of AgNPsand AgNP-H_2O_2 treated cells were dispensed on clean slides (3 slides for each treatment). Slides were air-dried, methanol fixed and rehydrated in descending concentrations of alcohol (100%, 90%, 75%and 50%). Slides were washed with distilled water for 5 mins. The slides wereimmersed in filtered hematoxylin stain for 3 mins and washed with distilled water twice followed by immersion in filtered eosin stain for 5 seconds and washed with distilled water. Dried slides were immersed in xylene followed by mounting with Canada balsam.The coverslips were added to each slide and left to air dry. Microscopic fields (100X) were photographed using a digital camera (Canon-Japan), connected to a light microscope. The photomicrographs were qualitatively evaluated for the presence of morphological criteria of apoptosis which were analyzed using image analysis software.

Statistical analysis

All experiments were performed in three independent tests. Data were presented as the mean ±standard deviation (SD) and analyzed using one-way analysis of variance (ANOVA). The results were considered statistically significant at probability <0.05.

Results

Cell viability assay

The cytotoxicity of AgNPs and AgNP-H_2O_2 following 24 hrs treatment on Vero cells was determined using MTT assay.Recorded data revealeda dose dependent increased cellular viability along with decreasing the concentrations of AgNPs and AgNP-H_2O_2 till reaching 100% viability at a concentration of 2.75 and 0.4 µM/ml, respectively. The observed IC_{50} value of AgNP-H_2O_2 was significantly (0.864 ± 0.05 µM/ml) lower (P<0.05) than in case ofAgNPs (7±0.20 µM/ml) (Figure 1).

Biochemical analysis

Regarding the evaluation of the Lactate dehydrogenase B (LDH-B) and reactive oxygen species (ROS)levels following treatment with AgNPs and AgNP-H_2O_2, it was found that LDH-B showed a significantly decreased values compared with its values in untreated cell control (P<0.05). In the meantime,results showed a significantly elevated levels of ROS in AgNPs and AgNP-H_2O_2 treated cells compared tountreated cell control (Figure 2).

Antiviral activity

Assessment of the antiviral potential of AgNPs and AgNP-H_2O_2 against HSV-1 showed a significant (P<0.05) depletion in HSV-1 infectivity titer recording 2.84 $\log_{(10)}$ /0.1 ml (59.79%) and 3.62 $\log_{(10)}$/0.1 ml (76.21%) reduction in case of the application of the tested nanoparticles prior to viral infection, respectively. On the other hand, lower depletion in the viral infectivity titer was observed post treatment of infected cells with AgNPs and AgNP-H_2O_2, where the reduction was in the order of 0.75 $\log_{(10)}$/0.1 m (15.79%) and 1.62 log (10) /0.1 ml (34.11%), respectively. In the mean time, the reduction in HSV-1 titer was higher in IFN-α2a pretreated cells (3.25 $\log_{(10)}$ /0.1 ml; 68.42%) compared to the recorded reduction (1.62 $\log_{(10)}$/0.1 ml; 29.45%) post treatment of the virus infected cells with IFN-α2a. Similarly, pretreatment of Vero cells with AgNPs and AgNP-H_2O_2 resulted in a significant (P<0.05) reduction in VSV infectivity titer with higher antiviral potential in case of AgNP-H_2O_2 (2.75 $\log_{(10)}$/0.1 ml; 57.89%) compared to AgNPs (1.75 $\log_{(10)}$/0.1 ml; 36.84%). While, the direct application ofAgNPs and AgNP-$H_2O_2$to VSV infected cells showed lower viral inhibitory activity, where the recorded depletion rate was in the order of 1.09 $\log_{(10)}$/0.1 ml (22.95%) and 1.75 $\log_{(10)}$/0.1 ml (36.84%), respectively. Concurrently, IFN-α2a pretreated cells showed a significant (P<0.05) reduction in VSV infectivity titer

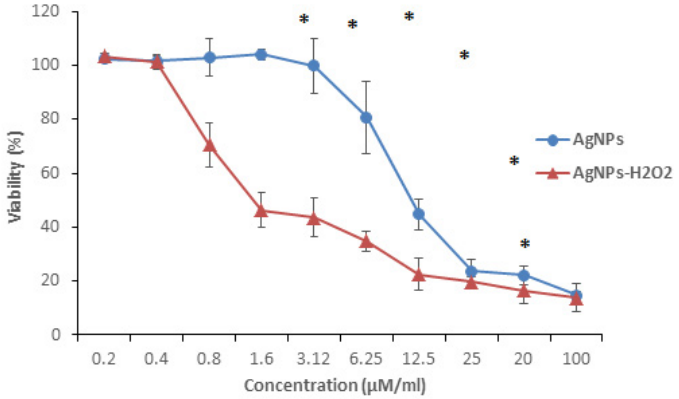

Figure 1. Evaluation of cytotoxicity of AgNPs and AgNP-H_2O_2 showing a significant reduction in cellular viability post Vero cells treatment with AgNP-H_2O_2 compared to AgNPs. *: Statistically significant difference.

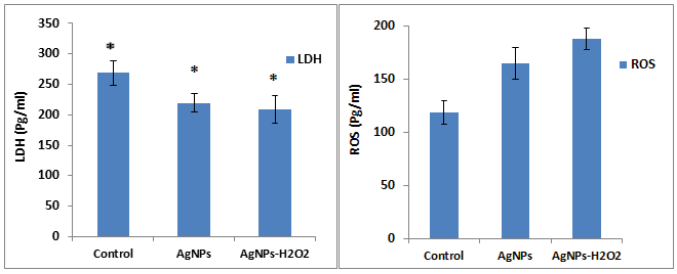

Figure 2. Evaluation of Lactate dehydrogenase enzyme (LDH-B) and Reactive oxygen species (ROS) in AgNPs and AgNP-H2O2 treated cells using ELISA. *: Statistically significant difference.

compared to its value in case of direct application of IFN-α2a post infection, where therecorded depletion rate wasin the order of 3.75log $_{(10)}$/0.1 ml (78.95%) and 0.75 log $_{(10)}$/0.1 ml (15.79%), respectively (Figure 3).

Recorded data concerning the antiviral activity of AgNPs,AgNP-$H_2O_2$and IFN-α2a against MERS-CoV revealed that AgNP-H_2O_2 and IFN-α2a showed a significant higher ($P<0.05$) antiviral potential compared to that observed in case of AgNPs. Results showed that thepercentage reductionin the plaque forming units was in the order of 33.3%,66.6% and 87.5%in case of treatment with AgNPs, AgNP-H_2O_2 and IFN-α2a,respectively.

MxA and 2'-5'-OAS gene expression levels

The expression level ofantiviral molecular markers namelyMxA and 2'-5'-OASgenes post cellular treartment with AgNPs, AgNP-$H_2O_2$and IFN-α2a showed a statistically significant ($P<0.05$) elevated expression levels of both genes compared totheir levels in untreated cell control. The expression level of MxA gene was increased in the order of 2.85,3.87and 5.66 fold post treatment withAgNPs, AgNP-H_2O_2 and IFNα2a, respectively compared tocontrol. In the same time, 2'-5'-OAS recorded an elevation of the expression level in the order of 3.56, 4.57 and 7.58 fold, respectively. Data also indicated a significantly elevated expression levels post treatment with AgNP-H_2O_2 and IFN-α2a compared to AgNPs treated cells (Figure 4).

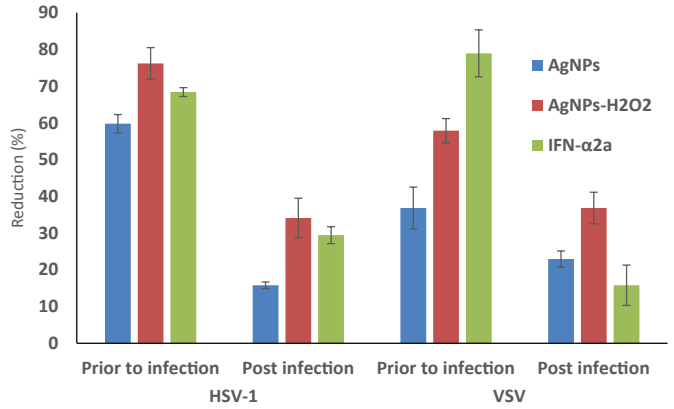

Figure 3. Assessment of the antiviral potentials of AgNPs, AgNP-H_2O_2 and IFN-α2a against HSV-1 and VSV using two different methods (pre and post viral infection).

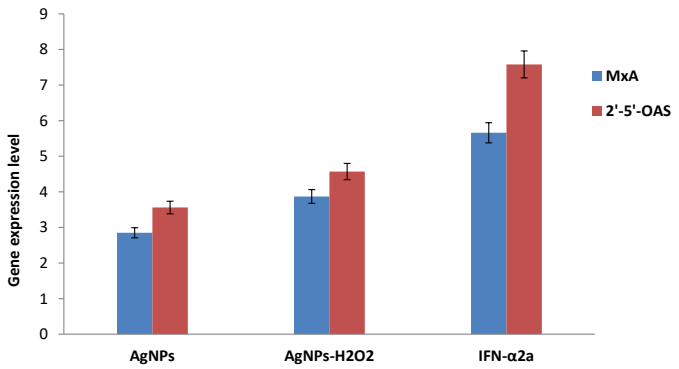

Figure 4. Assessment of MxA and 2'-5'-OAS gene expression levels post Vero cell treatment with AgNPs, AgNP-H_2O_2 and IFN-α2a using real time PCR.

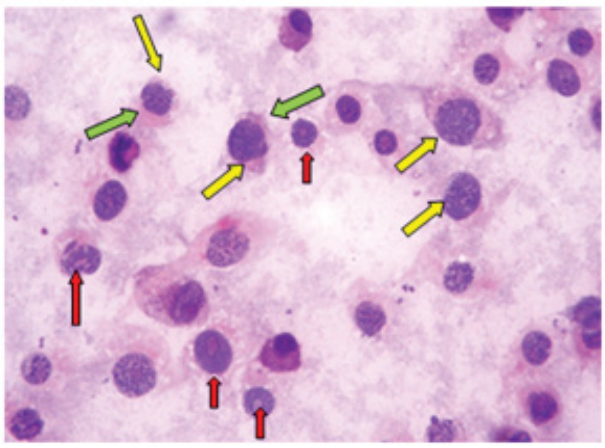

Figure 5a. AgNPs treated cells showing swollen cells and swollen nuclei with mixed euochromatin and heterochromatin (Yellow arrows) and ruptured cell membranes (Green arrows). Intranuclear eosinophyllic structures (Red arrows).

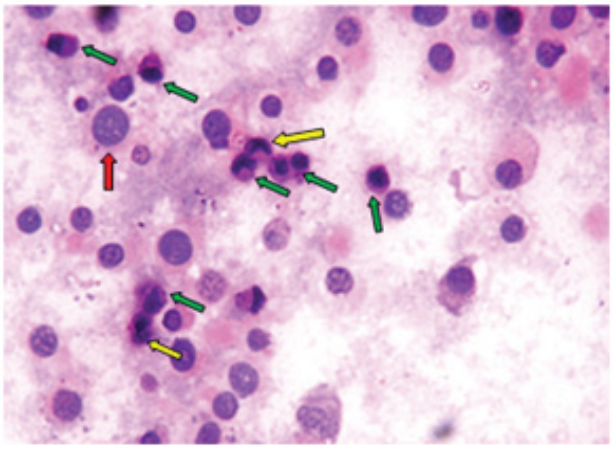

Figure 5b. AgNP-H_2O_2 treated cells showing shrunken apoptotic cells (Green arrows) with peripheral condensation of chromatin (Yellow arrows) and necrotic swollen cell with mixed euochromatin and heterochromatin and ruptured cell membrane (Red arrow).

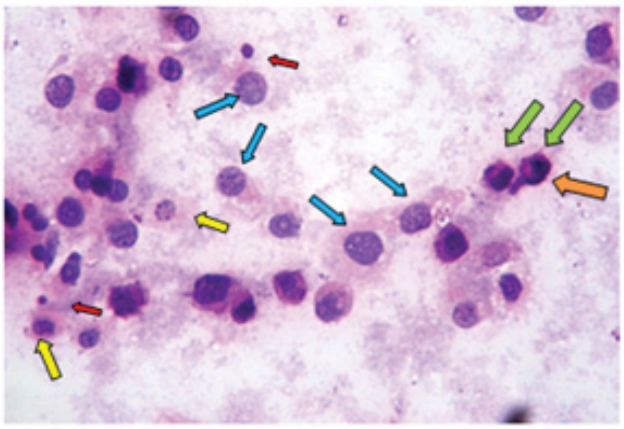

Figure 5c. AgNP-H_2O_2 treated cells showing necrotic swollen cells and swollen nuclei with mixed euochromatin and heterochromatin and ruptured cell membranes (Blue arrows). Shrunken apoptotic cells (Green arrows) with peripheral condensation of chromatin (Orange arrow). Secondary necrotic cells with peripheral condensation of chromatin and ruptured cell membranes (yellow arrows). Apoptotic bodies (Red arrows).

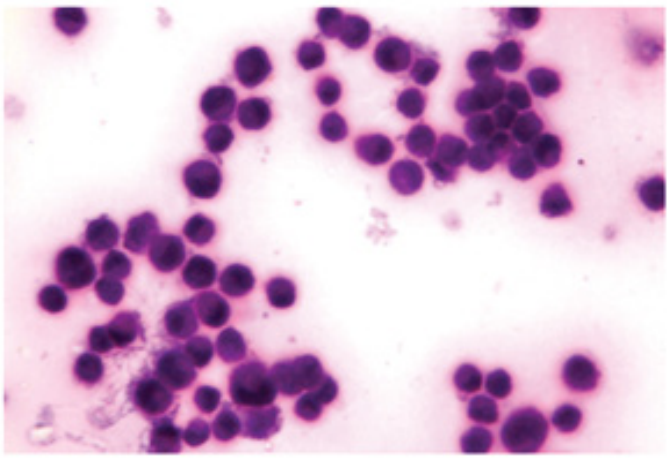

Figure 5d. Untreated control cells with regular hyperchromatic nuclei and nuclear pleomorphism.

Pathological changes

Assessment of pathological changes usinghematoxylin and eosin staining post treatment with AgNPs showed swollen Vero cells as well as swollen nuclei with mixed euochromatin and heterochromatin, ruptured cell membranes and intranuclear eosinophyllic structures. AgNP-H_2O_2 treated cells exhibited pathologic abnormalities similar to that observed in case of AgNPs treated cells with additional apoptotic features of shrunken cells, shrunken nuclei and peripheral condensation of chromatin. On the other side, untreated control cells showed regular cells with hyperchromatic nuclei and nuclear pleomorphism (Figure 5).

Discussion

Nanotechnology-based medical applications are progressively increased.The present study aimed to evaluate the antiviral potential of silver nanoparticles against different virus models including DNA, RNA viruses and MERS-CoVas well. Cytotoxicity of AgNPs and AgNP-H_2O_2 were evaluated using Vero cells and revealed that the toxic effect of both particles was in a concentration dependent manner with higher cytotoxicity in case of AgNP-H_2O_2compared toAgNPs. Concerning the cytotoxicity of AgNPs, Nowrouzi*et al*., (2010) [32] reported that the IC_{50} of AgNPsagainst hepatoma ($HepG_2$) cells was 2.75 and 3.0 mg/lusing XTT and MTT, respectively. Also, Zhang *et al*., (2017) reported that the IC_{50} value of AgNPs against human laryngeal carcinoma(HEp-2) cells was 2.24 µg/ml post 24 hrs incubation [33]. In another study, Paknejadi *et al*., (2018) reported that the synthesized AgNPs showed a significant toxicity to normal human skin fibroblast cell line using MTT assay in a concentration and time dependent manner. The calculated IC_{50} values were 30.64 and 14.98 µg/ml post 24 and 48 hrs treatment, respectively [34].The variation in AgNPs cytotoxicity results between different studies is related to several factors such as the difference insize and surface coating of nanoparticles [35] in addition to the diverse sensitivity of cells towards the same nanoparticles [36]. It was also reported that the intracellular concentration of AgNPs is the main determinant of their toxicity to mammalian cell lines due to the accumulation of AgNPs in the cytoplasm and nuclei of treated cells. Also, another study found that the cytotoxicity AgNPs is associated with reduction in the celluar ATP content due to induction of mitochondrial damage ae well as elevated production of reactive oxygen species (ROS) in a concentration-dependent manner [37].This consequently resulted in induction of intracellular oxidative stress [35] which was in accordance with the current results, where treatment of Vero cells with AgNPs and AgNP-$H_2O_2$was accompanied by elevated levels of ROS compared to control cells. Similaily, quantification of the released lactate dehydrogenase (LDH) is a well-established assay for assessment of cellular viability,where LDH is released from cells in response to cellular damage [38]. Our study showed that cytotoxic effect of AgNPs and AgNP-H_2O_2 may be related to cellular damage due to the recorded reduced levels of LDH post treatment with AgNPs and AgNP-H_2O_2 compared to untreated control. On the other hands, AgNPs and AgNP-H_2O_2didn't show cytotoxic potential to Vero cells and investigation of the antiviral activities of these particles was carried out using the non-toxic concentrations of both types of nanoparticles.

It is important to point out that the use of metal nanoparticles provides an exciting opportunity for the development of novel antiviral therapies. Since metals may attack a wide range of viral targets with lower possibilities of resistance compared to conventional antiviral agents. Also, there were different trials performed for the production and development of silver nanoparticles for their application as antiviral therapeutic agents. It was also demonstrated that AgNPs are a potential enhanser for cellular release of H_2O_2 [39].Thus, our study investigated not only the antiviral potential of AgNPs but also the antiviral activity of hydrogen peroxide combined silver nanoparticles (AgNP-H_2O_2) were examined.A study demonstrated that silver ions may suppress the viral attachment to cellular receptors via their interaction with the viral receptors resulting in an alteration in the viral epitopes.Results of our study were in accordance with another study recordedthatAgNPs and silver nitrate showed to have antiviral activities aganist feline calicivirus and the murine norovirus [40]. It was also observed that the use of AgNPs alone could inhibit cell associated HIV-1 infection, whereas the application of AgNPs and monoclonal antibody mix aganist HIV-1 could inhibit the cell associated HIV-1 infection in an additive way [41]. More over, another study reported that AgNPs were effective against different types of viruses including HIV, Hepatitis B virus, herpes simplex virus, respiratory syncytial virus(RSV) and monkeypox virus. They attributed this activity to the probaility that metal ions may interact with variable viral targets [42].

Recently, it was reported that theantivitral potential of AgNPs is attributed to the natureof the virus structure. For example,respiratory syncytial virus (RSV) harbors two surface glycoproteins (F and G) those are necessary for the initial phases of infection, making them essential targets for antiviral therapyagainst this virus. The study found that the broad-spectrum antiviral activities of AgNPs against respiratory viruses was mediated via attaching to viral glycoproteins thus preventing the entry of the virus into the host cell. Treatment with AgNPs resulted in reduction in the viral replication in epithelial cell lines as well as in experimentally infected BALB/c mice with significant reduction in pro-inflammatory cytokines and chemokines [43]. In the same sequence, another study demonstrated that AgNPs interact with HIV-1 virus through binding to the gp120 glycoprotein knobs and consequently prevent the virus from binding to host cells

[44]. These results were in agreement with the present study, where we recordedelevated antiviral potentials of AgNPs and AgNP-H_2O_2 againstHSV-1 and VSV when the particles were applied to Vero cells prior to viral infection compared to the inhibitory potentials when the cells were treated with the particles post infection. This suggested that the antiviral activity of the tested particles is associated with early stages of viral infection such as the events related to viral attachment and enteryto the host cells rather than inhibiting the late stages of viral infection. Additionally, therecorded results shed light on the potential of investigating the avtiviral activity of AgNP-H_2O_2, where they exhibited higher antiviral activities against HSV-1, VSV and MERS-CoV compared to that recorded in case of testing AgNPs alone, despite that the tested concentration of AgNP-H_2O_2(0.4 μM/ml) was much lower than that applied in case of AgNPs (2.75 μM/ml).To the best of our knowledge, this is the first study that reported the antiviral potential of combining silver nanoparticles with hydrogen peroxide againstHSV-1, VSV and MERS-CoV compared to silver nanoparticles alone as well as the standard interferon.

Regarding the AgNPs antiviral mehansim of action, there was a relation between inhibitory activity of these particle either alone or in combination with H_2O_2 against HSV-1, VSV,MERS-CoV and the expression profiles Mx-A and 2'-5'-OASgenes.This was in compliance witha study which reported that VSV transcription was inhibited by MxA protein [45]. It was also reported that recombinant MxA protein synthesized in *Escherichia coli*prevents in-vitro RNA synthesisof VSV and influenza A virus [46]. Many other studies have also demonstrated the inhibitory potentials of MxA protein against thogoto virus [47], bunya, phlebo, and hanta viruses [48] as well as puumala and tula hantaviruses [49] in addition to

La-Crosse virus [50]. Regarding the antiviral mechanism of MxA protein, it was found that Vero cells transfected with MxA showed potentially decreased expression levels of dugbe virus (DUGV) antigen which may reflect the inhibitory effect of MxA on DUGV replication [51]. It was also reported that the type of the virus and the host cell are critical determinants in the antiviral activities of MxA protein, where MxA inhibited measles virus in the human mononuclear (U937) and glioblastoma (U87) cell lines but not in Vero or Hep-2 cells [51]. In addition, respiratory syncytial virus (RSV) wasn't inhibited by MxA either in U87 or Vero cells [53], whereas the infectivity of murine pneumovirus was abolished by transgenic mouse cells expressing bovine Mx protein [54].

It is essential to recognize the importanceof naturally developed IFN k.type -1 in the enhancement of the antiviral activity, where IFN type-I plays a crucial role in the first line of defense against viral infections. It was found that the expression of the IFN-inducible Mx gene was up-regulated post Giant Salamander Iridovirus (GSIV) infection [55] resulting in an inhibitory effect on GSIV replication [56]. Additionally, 2′,5′-oligoadenylate-synthetasedirected ribonuclease is considered an essential pathway in IFN-mediated antiviral response. This pathway participates in blocking the viral transcription, degradation of the viral RNA via stimulation of cellular ribonuclease (RNase), inhibition of translation and modification of protein function to control all steps involved in viral replication [57].

Concerning the role of 2′,5′-oligoadenylate synthetase as antiviral marker, our results were complied with [58] reporting that it is an extensively characterized enzyme induced by interferon and it is important for an effective antiviral response particularlyin the presence of double-stranded RNA structures like viral genomes. It was further demonstrated that NS1 protein of influenza A virus confers antiviral protection through inhibiting the IFN-induced 2′-5′-oligoadenylate-synthetase/RNase pathway. Additionally, it was found that the antiviral state could be verified by production of three well-defined human proteins playing an essential role in regulating virus infection, namely double-stranded RNA-activated protein kinase (PKR), 2′,5′-OAS and MxA [59].

Recorded data concerning the pathological changes developed in Vero cells post AgNPs and AgNP-H_2O_2 treatment was in accordance to [60] despite the use of variable cell line and the method of treatment. This study investigated the effect of electroporation of Hep-2 cancer cells in a presence of gold nanoparticles (EP Au-NPs) as well as the effect of exposure of these cells to extremely-low frequency electromagnetic field (ELFEMF). It was observed that treated cells showed numerous apoptotic bodies, necrotic cells with altered euchromatin and heterochromatin as well as ruptured cell membrane.The ultrastructural alterations post treatment of hepato-cellular carcinoma (HepG2) cells with silver nanoparticles were examined in another study using transmission electron microscopy. It was reported that AgNPs-treated cells showed disruption of nuclear membranes, formation of blebbed nuclei and accumulation of autophagic vacuoles containing destructed organelles. Inaddition to the observationof several swollen lipid dropletsin the cytoplasm as well as damage of other cytoplasmic organelles including swelling ofthe mitochondria along withfading of mitochondrial inner membrane [61]. Also, another study reported that subjecting human keratinocyte (HaCaT) cells to AgNPs resulted in increased number of autophagosome and many cells exhibited features of apoptosis [62].

Finally, it could be concluded that both AgNPs and AgNP-$H_2O_2$are potentially active antiviral agents,where their activitiesarealmost equivalent to that of the reference interferon. The combination of H2O2withAgNPsenhanced the activity of free AgNPsagainst both DNA and RNA virus models, namely HSV-1, VSV and MERS-CoV. The elevated expression levels of Mx-A and 2′,5′-OAS genes post treatment with test drugs presented a respectable markerfor their antiviral potentials. On the other hand, the cytotoxicty profile, biochemical and cytopathological changes accompanied by the application of these nanoparticels should be taken into consideration in addition to deep monitoring of the related changes on the sub-cellular level.

References

1. Pal S, Tak YK, Song JM. "Does the antibacterial activity of silver nanoparticles depend on the shape of the nanoparticle? A study of the gram-negative bacterium *Escherichia coli*." *Appl Environ Microbiol* 73(2007):1712-1720.
2. Sondi I, Salopek-Sondi B." Silver nanoparticles as antimicrobial agent: a case study on E. coli as a model for Gram-negative bacteria."*J Colloid Interface Sci* 275(2004):177-182.
3. Morones JR, Elechiguerra JL, Camacho A and Holt K, et al. "The bactericidal effect of silver nanoparticles." *Nanotechnology* 26(2005):2346.
4. Gajbhiye M, Kesharwani J, Ingle A and Gade A, et al. "Fungus-mediated synthesis of silver nanoparticles and their activity against pathogenic

fungi in combination with fluconazole." *Nanomedicine* 5 (2009):382-326.

5. Liau SY, Read DC, Pugh WJ and Furr JR, et al. "Interaction of silver nitrate with readily identifiable groups: relationship to the anti-bacterialaction of silver ions." *Lett Appl Microbiol* 25 (1997):279-283.
6. Elechiguerra JL, Burt JL, Morones JR and Camacho-Bragado A, et al. "Interaction of silver nanoparticles with HIV-1." *J Nanobiotechnology* 3(2005):1.
7. Trefry JC, Wooley DP. "Rapid assessment of antiviral activity and cytotoxicity of silver nanoparticles using a novel application of the tetrazolium-based colorimetric assay." *J Virol Methods 183 (*2012):19-24.
8. Lu L, Sun RW, Chen R and Hui CK, et al." Silver nanoparticles inhibit hepatitis B virus replication." *Antivir Ther* 13 (2008):253.
9. Baram-Pinto D, Shukla S, Perkas N and Gedanken A, et al. "Inhibition of herpes simplex virus type 1 infection by silver nanoparticles capped with mercaptoethanesulfonate." *Bioconjugate Chem* 20 (2009):1497-1502.
10. Sun L, Singh AK, Vig K, Pillai SR and Singh SR." Silver nanoparticles inhibit replication of respiratory syncytial virus." *J Biomed Nanotechnol* 4(2008):149-158.
11. Rogers JV, Parkinson CV, Choi YW and Speshock JL, et al. "A preliminary assessment of silver nanoparticle inhibition of monkeypox virus plaque formation." *Nanoscale Res Lett* 4(2008):129.
12. Speshock JL, Murdock RC, Braydich-Stolle LK, Schrand AM and Hussain SM. "Interaction of silver nanoparticles with Tacaribe virus."*J Nanobiotechnology*. 8(2010):1-9.
13. Mehrbod P, Motamed N, Tabatabaian M and Soleimani ER, et al ."In vitro antiviral effect of "nanosilver" on influenza virus." *J Pharm Sci* 17(2009):88-93.
14. Xiang DX, Chen Q, Pang L, Zheng CL. "Inhibitory effects of silver nanoparticles on H1N1 influenza A virus in vitro." *J Virol Methods* 178(2011):137-142.
15. Wise Sr JP, Goodale BC, Wise SS and Craig GA, et al." Silver nanospheres are cytotoxic and genotoxic to fish cells." *Aquat Toxicol* 97(2010):34-41.
16. Navarro E, Piccapietra F, Wagner B, Marconi F and Kaegi R et al. "Toxicity of silver nanoparticles to Chlamydomonasreinhardtii." *Environ Sci Technol* 42(2008):8959-64.
17. Braydich-Stolle LK, Lucas B, Schrand A, Murdock RC and Lee T et al. "Silver nanoparticles disrupt GDNF/Fyn kinase signaling in spermatogonial stem cells."*Toxicol Sci* 116(2010):577-589.
18. Matyjas-Zgondek E, Bacciarelli A, Rybicki E and Szynkowska MI, et al. "Antibacterial properties of silver-finished textiles." *Fibres Text East Eur* 5 (2008):101-107.
19. Filipowska B, Rybicki E, Walawska A and Matyjas-Zgondek E. "New method for the antibacterial and antifungal modification of silver finished textiles." *Fibres Text East Eur* 4(2011):124-128.
20. Murugadoss A, Chattopadhyay A. "A 'green'chitosan–silver nanoparticle composite as a heterogeneous as well as micro-heterogeneous catalyst." *Nanotechnology* 19(2007):015603.
21. Sanpui P, Murugadoss A, Prasad PD and Ghosh SS, et al. "The antibacterial properties of a novel chitosan–Ag-nanoparticle composite." *Int J Food Microbiol* 124(2008):142-146.
22. Fayaz AM, Ao Z, Girilal M, Chen Land Xiao X et al." Inactivation of microbial infectiousness by silver nanoparticles-coated condom: a new approach to inhibit HIV-and HSV-transmitted infection." *Int J Nanomedicine* 7(2012):5007.
23. Mentel' R, Shirrmakher R, Kevich A, Dreĭzin RS and ShmidtI. "Virus inactivation by hydrogen peroxide." *Virol J* 6(1977):731-3
24. Essam K, Mohsen R, Ismail EA, Mohamed AF. "In Vitro Preparation of H2O2 Inactivated Rabies Vaccine and Related Immunogenicity." *Int J Respir Pulm Med* 3(2018):1-8.
25. Ramadan RH, Mohamed AF, El-Daim A, Mohamed S. "Evaluation of antiviral activity of honeybee venom on DNA and RNA virus models." *Egypt Acad J Biol.Sci* 2(2009):247-58.
26. El-Garhy FM, Badr DA, Mohamed AF." Assessment of Anti-cancer and Anti-viral potential of Pomegranate peel extract against human Prostate, and Larynx cancer cell lines: In-Vitro Study." *Annu Rev Cancer Biol* 7(2017):65-73
27. Reed LJ, Muench H. " A simple method of estimating fifty per cent endpoints."*Am J Hyg*27(1938):493-7.
28. Sala A, Ardizzoni A, Ciociola T, Magliani W and Conti S, Blasi E et al." Antiviral activity of synthetic peptides derived from physiological proteins."*Intervirology*. 61(2018):166-73.
29. Yonys KA, Saber SA, El-Fiky AA, Mohamed AF." In Vitro Evaluation of Antiviral/Virucidal Activity of NajaNubiae (Elapidae) Venom Against Rift Valley Fever and Herpes Simplex Virus Type-1 (HSV-1) Using Cell Culture." *Egypt J Hosp Med* 77(2019):4963-4969.
30. Baer A, Kehn-Hall K. "Viral concentration determination through plaque assays: using traditional and novel overlay systems." *J Vis Exp* 4(2014): e52065.
31. Sholqamy M I, Abd-ElHamid ES, Mostafa El-Bolok AH and Mohamed AF, et al. "Monitoring the Anticancer Effects of Two Different Gold Nanostructures Shapes towards Hep-2 Cells." *Int J Med Nano Res* 6(2019):028
32. Nowrouzi A, Meghrazi K, Golmohammadi T, Golestani A and Ahmadian S et al." Cytotoxicity of subtoxicAgNP in human hepatoma cell line (HepG2) after long-term exposure." *Iran Biomed J* 14(2010):23.
33. Zhang Y, Lu H, Yu D, Zhao D. "AgNPs and Ag/C225 exert anticancerous effects via cell cycle regulation and cytotoxicity enhancement." *J Nanomater* (2017).
34. PaknejadiM, Bayat M, Salimi M, Razavilar V."Concentration-and time-dependent cytotoxicity of silver nanoparticles on normal human Skin fibroblast cell line." *Iran Red Crescent Med J* 20(2018).
35. Ivask A, Visnapuu M, Vallotton P, Marzouk ER and Lombi E et al. "Quantitative multimodal analyses of silver nanoparticle-cell interactions: Implications for cytotoxicity." *Nano Impact* 1(2016):29-38.
36. Sahu D, Kannan GM, Tailang M, Vijayaraghavan R." In vitro cytotoxicity of nanoparticles: a comparison between particle size and cell type."*J Nanosci* (2016).
37. Sambale F, Wagner S, Stahl F, Khaydarov RR and Scheper T et al. "Investigations of the toxic effect of silver nanoparticles on mammalian cell lines." *J Nanomater* (2015).
38. Kaja S, Payne AJ, Naumchuk Y, Koulen P. "Quantification of lactate dehydrogenase for cell viability testing using cell lines and primary cultured astrocytes."*Curr Protoctoxico* 72(2017):2-6.
39. Hsiao IL, Hsieh YK, Chuang CY, Wang CF and Huang YJ. "Effects of silver nanoparticles on the interactions of neuron-and glia-like cells: Toxicity, uptake mechanisms, and lysosomal tracking." *Environ Toxicol Chem* 32(2017):1742-53.
40. Castro-Mayorga JL, Randazzo W, Fabra MJ, Lagaron JMand Aznar R et al." Antiviral properties of silver nanoparticles against norovirus surrogates and their efficacy in coated polyhydroxyalkanoates systems."*LWT-Food Science and Technology*. 79(2017):503-10.
41. Lara HH, Ixtepan-Turrent L, Treviño EN, Singh DK." Use of silver nanoparticles increased inhibition of cell-associated HIV-1 infection by neutralizing antibodies developed against HIV-1 envelope proteins."*J Nanobiotechnology* 9(2011):1-9.

42. Galdiero S, Falanga A, Cantisani M, Ingle A and Galdiero M, et al. “Silver nanoparticles as novel antibacterial and antiviral agents.”*Nanobiomedical research: Fundamentals, Applications and Recent Developments: Materials for Nanomedicine* 1(2014):565-594.
43. Morris D, Ansar M, Speshock J, Ivanciuc T and Qu Y et al. “Antiviral and Immunomodulatory Activity of Silver Nanoparticles in Experimental RSV Infection.” *Viruses* 11(2019):732.
44. Elechiguerra JL, Burt JL, Morones JR, Camacho-Bragado A and Gao X. “Interaction of silver nanoparticles with HIV-1.” *J Nanobiotechnology* 3(2005):1-0.
45. Schwemmle M, Weining KC, Richter MF, Schumacher B and Staeheli P. "Vesicular stomatitis virus transcription inhibited by purified MxA protein.” *Virology* 206(1995):545-54.
46. Ponten A, Sick C, Weeber M, Haller O and Kochs G.” Dominant negative mutants of human MxA protein: domains in the carboxyterminal moiety are important for oligomerization and antiviral activity.”*J Virol* 71(1997):2591-2599.
47. Frese M, Kochs G, Meier-Dieter U, Siebler J and Haller O. “Human MxA protein inhibits tick-borne Thogoto virus but not Dhori virus.”*J Virol* 69(1995):3904-3909.
48. Frese M, Kochs G, Feldmann H, Hertkorn C, and Haller O.” Inhibition of bunyaviruses, phleboviruses, and hantaviruses by human MxA protein.” *J Virol* 70(1996):915-923.
49. Kanerva M, Melén K, Vaheri A, Julkunen I. "Inhibition of puumala and tula hantaviruses in Vero cells by MxA protein.” *Virology* 224(1996):55-62.
50. Reichelt M, Stertz S, Krijnse-Locker J, Haller O and Kochs G. “Missorting of LaCrosse virus nucleocapsid protein by the interferon-induced MxAGTPase involves smooth ER membranes.” *Traffic* 5(2004):772-84.
51. Bridgen A, Dalrymple DA, Weber F, Elliott RM. “Inhibition of Dugbenairovirus replication by human MxA protein.”*Virus Research* 99(2004):47-50.
52. Schneider-Schaulies S, Schneider-Schaulies J, Schuster A and Bayer M, et al.”Cell type-specific MxA-mediated inhibition of measles virus transcription in human brain cells.” *J Virol* 68(1994):6910-7.
53. Atreya PL, Kulkarni S. “Respiratory syncytial virus strain A2 is resistant to the antiviral effects of type I interferons and human MxA.” *Virology* 261(1999):227-241.
54. Dermine M, Desmecht D.”In vivo modulation of the innate response to pneumovirus by type-I and-III interferon-induced Bostaurus Mx1.” *J Interferon Cytokine* 32(2012):332-337.
55. Chen Q, Ma J, Fan Y, Meng Y and Xu J et al.” Identification of type I IFN in Chinese giant salamander (Andriasdavidianus) and the response to an iridovirus infection.” *Mol Immunol* 65(2015):350-359.
56. Liu Y, Li Y, Zhou Y, Jiang N and Fan Y et al. “Characterization, Expression Pattern and Antiviral Activities of Mx Gene in Chinese Giant Salamander, Andriasdavidianus.” *Int J Mol Sci* 21(2020):2246.
57. Sadler AJ, Williams BR. “Interferon-inducible antiviral effectors.”*Nat. Rev. Immunol*. 8(2008):559-68.
58. Min JY, Krug RM. “The primary function of RNA binding by the influenza A virus NS1 protein in infected cells: inhibiting the 2′-5′ oligo (A) synthetase/RNase L pathway.”*ProcNatlAcadSci* 103(2006):7100-5.
59. Le Tortorec A, Denis H, Satie AP, Patard JJand Ruffault A et al. “Antiviral responses of human Leydig cells to mumps virus infection or poly I: C stimulation.” *Hum Reprod* 23(2008):2095-103.
60. Alshehri MA, Wierzbicki PM, Kaboo HF, Nasr MS and Amer ME et al.” In vitro evaluation of electroporated gold nanoparticles and extremely-low frequency electromagnetic field anticancer activity against Hep-2 laryngeal cancer cells.”*Folia Histochem. Cytobiol* 57(2019):159-167.
61. Sooklert K, Wongjarupong A, Cherdchom S and Wongjarupong N, et al. "Molecular and morphological evidence of hepatotoxicity after silver nanoparticle exposure: A systematic review, in silico, and ultrastructure investigation.” *Toxicol Res* 35(2019):257-270.
62. Perde-Schrepler M, Florea A, Brie I and Virag P, et al. "A Size-dependent cytotoxicity and genotoxicity of silver nanoparticles in cochlear cells in vitro.” *J Nanomater* (2019).

How to cite this article: Adel-El-bialy, Ahmed M. Mansour, Tamer M. M. Abuamara, Mohamed E. Amer and Rania I Shbel, et al. "Histopathological, Biochemical and Genetic Profile Post Application of Free Silver Nanoparticles and H_2O_2 Combined Form In Vitro Study." J Cytol Histol 12 (2021): 564.