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Investigating The Cytotoxic Effects Of Coated Silver Nanoparticles On Cancer Cervical Cells

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Abstract

Silver nanoparticles are taking on a greater role in cancer therapy and are beginning to overtake the current traditional approaches. This is because silver nanoparticles specifically target cancer cells, have fewer side effects, and have potent anti-cancer properties. A variety of biocompatible polymers such as hyaluronic acid and polyethyleneimine are being used for coating nanoparticles. Coating nanoparticles improves the stability of the nanoparticles (NP), impart biocompatibility, promote long-term circulation and to increase cellular uptake. This study was designed to investigate the cytotoxic effects of the silver nanoparticles on cervical cancer lines. The cytotoxic effects of silver citrate (Ag Citrate) and silver Polyvinylpyrrolidone (PVP) (Ag PVP) was tested on Hela cells. The cells were exposed to equal concentrations (25 µg/ml) of the nanoparticles for 24hrs. The change in morphology which could signal apoptosis was observed while caspase-3 and superoxide dismutase cytotoxic assay were carried out to analyze the type of cell death. The morphological changes showed that Zinc oxide (ZnO) the (positive control) completely killed the cells after 24hrs while Ag Citrate had more effects on the shape of the cells than Ag PVP. In the cytotoxicity assay, ZnO also induced more caspase-3 and superoxide dismutase activity than Ag Citrate and Ag PVP. Between the two nanoparticles, Ag Citrate induced more caspase-3 activity than Ag PVP. In terms of superoxide dismutase, Ag PVP induced more activity than Ag Citrate. These results do indicate that both the coated nanoparticles induce cell death in cancer cells.

Keywords: *Silver nanoparticles; coated nanoparticles; silver citrate; polyvinylpyrrolidone (PVP); HeLa cells; cervical cancer; cytotoxicity; apoptosis; caspase-3; oxidative stress*

Introduction

Silver nanoparticles (AgNPs) constitute have been widely used in biological, medical, and engineering science. Synthetic biologically active AgNPs, for example, possess broad spectrum antibiotic, antifungal, anticancer, anti-inflammatory, wound healing, antioxidant, and anti-diabetic activity (Xu et al., 2020).Modifying the surface of nanoparticles is important. As a result, it reduces the toxic properties of AgNPs and the initial stabilizing agents, prevents their aggregation, and increases their ability to target specific cells, specifically cancer cells (Isabirye et al., 2012). Nanomaterials could be coated with various materials to control their bioavailability, and toxicity (Auclair et al., 2019).It is possible to modify the surface of AgNPs and other nanoparticles to give them additional properties such as improved persistence and interaction with biological targets such as cancer cells and microorganisms. Furthermore, coatings could also affect the size and form of the NPs (Auclair et al., 2019). Nanoparticle surface properties can affect blood circulation as well. In response to their administration, NPs can bind to opsonin's serum proteins, including immunoglobulins and complement proteins that serve as receptors for macrophages (Helena et al., 2021). Therefore, opsonization determines if NPs remain in the bloodstream or if they are phagocytosed. Therefore, altering its surface can increase or decrease its blood circulation time (Helena et al., 2021).

With these considerations in mind, we chose to investigate the possible medical applications of silver nanoparticles by investigating the effects of coating on their cytotoxicity on cervical cancer cells. These AgNPs were coated with two different coatings; citrate and polyvinylpyrrolidone, both of which have a negative charge on their surfaces. We examined the toxicity of the compound by determining Caspase 3 and catalase activity as well as superoxide dismutase release and lipid peroxidation using colorimetric methods and fluorescence microscopy.

Methodology

Cell culture and exposure to silver nanoparticles

To examine the morphological changes, HeLa cells were cultured in 12 well plates at density of $1x10^5$ and incubated for 24 hrs. in a 37℃, 5% CO_2 incubator. The nanoparticles which were in solution were provided by a PhD student and were already characterized. They were sonicated before use to separate them. The cells were separately treated with 25 µg/ml of Ag PVP, Ag Citrate and (ZnO) as a positive control. Cells not treated with nanoparticles were used as a negative control and were all incubated. The cells were observed at 6hrs, 18hrs and 24hrs. The media was collected for a measurement of Caspase-3 activity and superoxide dismutase activity.

Biochemical tests

Caspase-3 activity assay

The generation of caspase 3 was assessed using the caspase- 3 assay kit (colorimetric) (ab39401) from Abcam (Abcam protocol, 2021). To the 6 microwell plate, 50 µL of the sample was added and 50 µL of reaction buffer to the control's wells. The samples were done in triplicates. A caspase reaction mix was prepared by mixing the 2 x Reaction Buffer with 0.5 µL of the DTT. To each sample a volume of 50 µL was added and also 5 µL of the 4mM of DEVD-*p*-NA substrate to a final concentration of 200 µM. The mixture was mixed well and incubated at 37℃ for 120 minutes. The absorbance of the samples was measured at a wavelength of 405nm, and the results were analyzed.

Superoxide dismutase assay

To determine the activity of the enzyme superoxide dismutase the superoxide dismutase assay kit (colorimetric) (ab65354) was used (Abcam Protocol, 2021). The action of xanthine oxidase produces superoxide anions in this experiment. The dismutation of the superoxide anion into hydrogen peroxide and oxygen is catalyzed by SOD. Water-soluble tetrazolium (WST) working solution is reacted with superoxide anions to produce a water-soluble formazan dye, which is identified by an increase in absorbance at 450 nm. The less formazan dye is generated, the higher the SOD activity in the sample. The cultivated cells were harvested in a micro 6 well plate. A volume of 200 µL of WST working solution was added to each well in triplicates. A volume of 20 µL of dilution buffer was added to blank 2 and 3 and 20 µL of enzyme working solution was also added to each sample and blank 1. The solution was mixed and incubated at 37℃ for 20 minutes. The absorbance readings were measured at 450 nm wavelength on a microplate reader. The SOD activity (inhibition rate %) was calculated using the following equation:

SOD Activity (inhibition rate %) = (Ablank1 – Ablank3) – (Asample – Ablank2) X 100
(Ablank1 – Ablank3)

Where A = absorbance

Results and Discussion

Morphological changes of HeLa cells after exposure to Ag Citrate and Ag PVP

The Hela cells were exposed to AgNPs for 24 hrs. The morphological changes were observed with an inverted microscope at x40 magnification. Notable changes in the morphology were observes after 18hrs of exposure to the nanoparticles. The cells showed increased granulation, debris collection and clump formation (Figure 4). Cells in negative control were in normal morphological shape at all the observed times of 0, 6, 18 and 24 hrs. Some clump formation and granularity were observed after 18 hrs. in both nanoparticles PVP had more visible morphological changes than the Ag Citrate. At 24 hrs, more than 50% of the cells had visible granularity, loss of morphology and more clumping. The positive control of ZnO killed the cells in the first 6 hrs proving to be more toxic.
Overall, the cells confluency decreased in both Ag PVP and Ag Citrate over the course of 24 hrs.

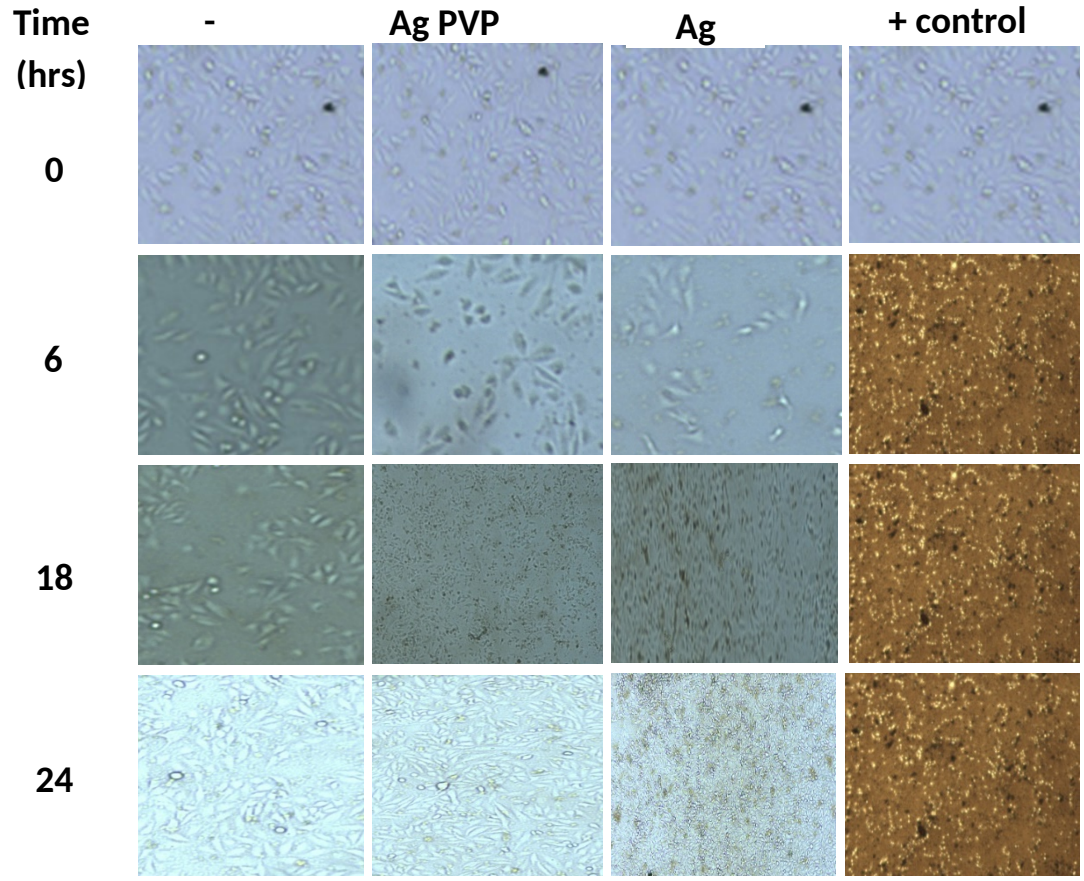


Figure 4; Morphological changes of HeLa cells at various time exposure to different nanoparticles. Hela cells were treated with AgNPs at a concentration of 25 µg/ml. The cells were observed under a microscope at x40 magnification.

The results have demonstrated that both Ag PVP and Ag Citrate induce morphological changes in HeLa cells after 18 hrs. of exposure. The morphological changes observed were granularity, clump formation and nuclear disintegration. In another study the same was observed with the human lung cancer cells (A549) when they were exposed at 50 g/mL of AgNPs for 24hrs; there was a considerable alteration in the cell's morphology, which is an indication of cell death and includes shrinkage(Xu et al., 2020).Upon the release of AgNPs into the cellular environment, an imbalance in the cellular homeostasis results. Based on the biological features of the cell this leads to apoptosis which is seen through morphological changes (Kovács et al., 2022).

Caspase-3 activity of HeLa cell lines in response to Ag PVP, Ag Citrate and ZnO

The extent of the activity of caspase-3, was determined by spectrophotometrically measuring the chromophore p-nitroaniline (p-NA) after cleavage from the labeled substrate DEVD-p-NA. Caspases are highly conserved cysteine proteases that play an important role in the apoptosis pathway regulation. Figure 5 demonstrates a rise in absorbance hence caspase 3 activity in HeLa cells exposed to the nanoparticles, indicating that AgNPs cause cell death by starting the apoptosis cascade. Absorbance is directly proportional to caspase-3 activity, meaning the more the absorbance recorded the higher the activity of caspase-3. The positive control (ZnO) has the highest absorbance (0.260). Ag Citrate (0.127) has a higher absorbance compared to Ag PVP (0.106), Figure 5.

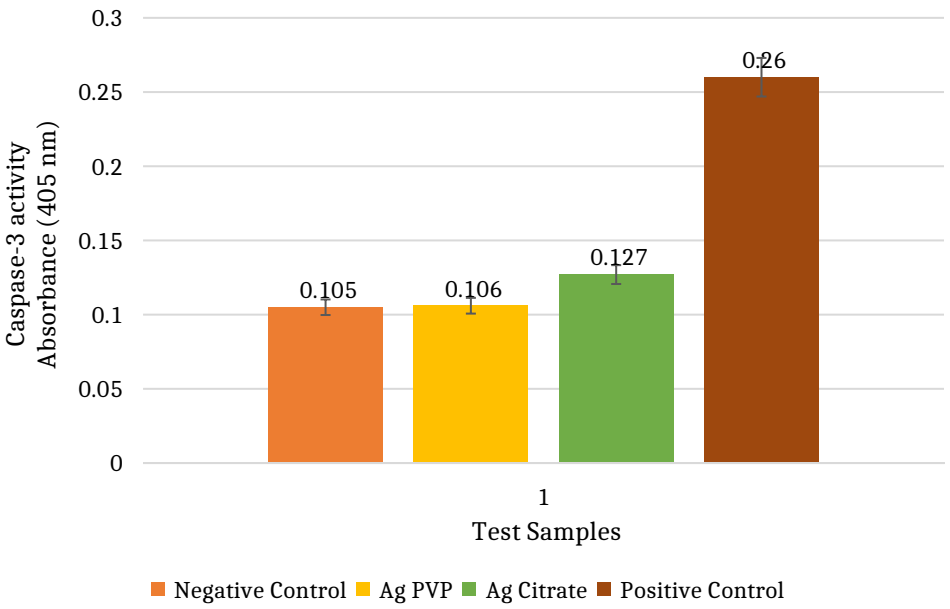


Figure 5; The caspase-3 activity absorbance values of Hela cells after exposure to AgNPs.

Capase-3 activity has always been observed as a biochemical marker for apoptosis (Köhler et al., 2002). Upregulation of casapase-3 activity was observed in the cells after 24 hrs of exposure to the AgNPs. The Hela cells exposed to ZnO (positive control) showed the highest absorbance hence more caspase-3 activity. According to Liu et al, (2017), ZnO nanoparticles cause oxidative stress in cancer cells, which has been discovered to be one of the causes of ZnO nanoparticles' cytotoxicity against cancer cells. Ag Citrate exposed cells had a higher caspase-3 activity compared to Ag PVP. This is shown by the higher absorbance of 0. 127.Our results agree with that found by (Bastos et al., 2016). According to Bastos et al (2016), the charged citrate coating has been shown to improve AgNP stability, and other investigations have found that citrate coated AgNPs may be highly cytotoxic to mammalian cells. The cytotoxicity of AgNPs is influenced by coating, size, and concentration (Behboodi et al., 2019). Citrate coated AgNPs have demonstrated to have more cytotoxic effects than PVP coated AgNPs as shown by that caspase-3 activity, (Figure 4).

Superoxide Dismutase activity assay of HeLa cell lines in response to Ag PVP, Ag Citrate and ZnO (control)

Figure 6 shows the activity of superoxide dismutase as measured by the colorimetric absorbance method. As the SOD activity decreases the absorbance increases, signaling low SOD activity. The positive control (ZnO) had the highest SOD activity (inhibition %). Between the two nanoparticles, Ag PVP had a higher inhibition % of 183% compared to Ag Citrate. The more inhibition a

compound has the more cytotoxic it is hence Ag PVP is more cytotoxic in terms of SOD activity. The cells exposed to Ag Citrate did however show a lower value than the negative control indicating that there was no superoxide dismutase activity in the Ag citrate treated cells.

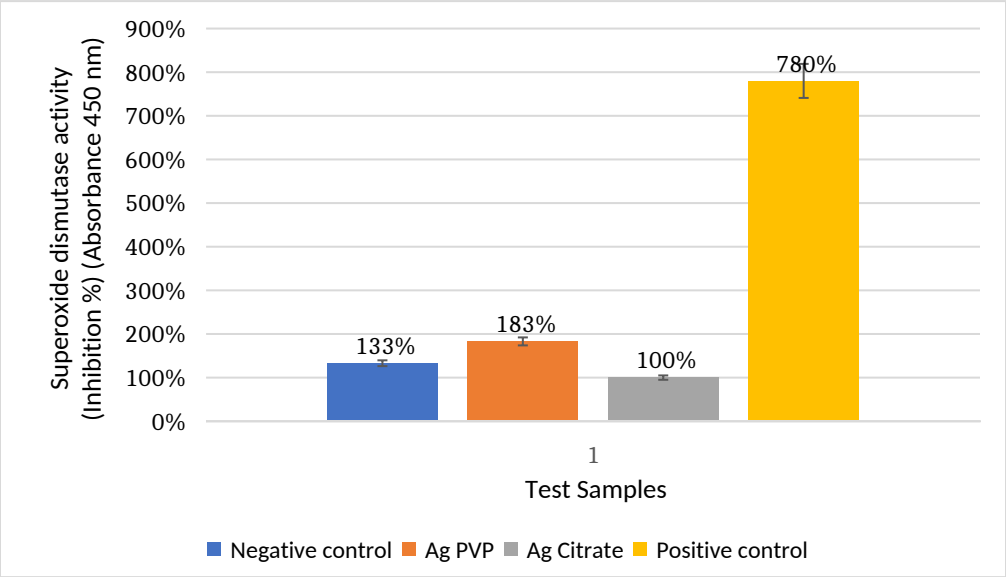


Figure 6; Superoxide dismutase activity of the nanoparticles with ZnO having the highest SOD activity Ag PVP had a higher inhibition % compared to Ag PVP.

In terms of superoxide dismutase activity, Ag PVP were found to be more cytotoxic as compared to Ag Citrate. One of the key mechanisms of nanoparticles is the generation of free radicals, which has been discovered in a wide spectrum of nanoparticles (Liu et al., 2012).It may cause oxidative stress, inflammation, and damage to proteins, membranes, and DNA. After a 24-hour exposure, the percentage of SOD inhibition increased in Ag PVP and Ag Citrate. This signifies that the cells were under oxidative stress.

Conclusion

When compared to currently available conventional medicinal drugs, AgNPs offer notable specificity with improved bioavailability. The anti-cancer activities of AgNPs have been studied in vitro using a variety of cell lines as model systems.

In summary, this experiment demonstrated that exposing cancer cells to AgNPs results in morphological changes and the activation of apoptosis. Apoptosis was activated through increased caspase-3 and superoxide dismutase activity. Comparing the two nanoparticles under investigation, Ag Citrate induced more caspase-3 activity than Ag PVP. However, Ag PVP induced

more superoxide dismutase compared to Ag Citrate. Ag citrate in fact had lower value of inhibition % than the negative control indicating no SOD activity in cells treated with Ag citrate. This difference was not due to the difference in concentration as both nanoparticles were at the same concentration of 25 µg/ml. The different coated AgNPs produced different effects regarding morphological changes, caspase-3 activity and superoxide dismutase. In future it will be more accurate to include other biochemical tests that are biomarkers of apoptosis such as the catalase and lipid peroxidation tests. There is also need to explore other mechanisms of AgNPs cytotoxicity apart from apoptosis.

Limitations of the study

The following are the limitations are encountered in this study.

a. Contamination of bacterial cells (4 times) with bacteria that hindered progress in the project
b. Unavailability of kits. Initially the research was supposed to investigate using four biochemical assays including lipid peroxidation and catalase activity assays

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