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CYTOTOXIC EFFECT OF SILVER NANOPARTICLES ON COLORECTAL ADENOCARCINOMA CELL LINE

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Silver nanoparticles (Ag-NPs) with antimicrobial activity are by far the most commercialized nano-compound. However, there is limited information about antitumor potential. Therefore, the present study was aimed to observe the effect of small-dimensional Ag-NPs (20 Nm) on human epithelial colorectal adeno-carcinoma (Caco-2) cells. Caco-2 cells were exposed to Ag-NPs with different concentrations (1–15 µg/mL) for 24 and 48 hrs. and cytotoxicity was analyzed by MTT assay and IC50 was calculated. In addition, flow cytometry was used to determine the effect of Ag-NPs on the cell cycle progression and on apoptosis. An MTT assay revealed that following exposure to Ag-NPs the viability of Caco-2 cells decreased, and the viability decreased further with increasing exposure time. The IC50 was 7.85 µg/ml and 4.92 µg/ml at 24 and 48 hrs. respectively after exposure. The flow cytometry technique revealed that Ag-NPs induced a significant increase in the percentage of cells in G1 phase of the cell cycle and of apoptotic cells. In conclusion, Ag-NPs exhibited dose- and time-dependent cytotoxicity, and these results have signposted that Ag-NPs were promising nanoparticles which could be a potential candidate in treating colorectal cancer.

INTRODUCTION

In the recent decades, nano-materials have been known for being one of the most used materials[1]. They have been described as the "the material of this century" for their exceptional designs and the characteristics of their combinations in contrast with bulk materials[1]. The use of nano-materials can help to develop products and fabrication processes with better features or new functionalities. Nano materials are known for having a widespread range of applications in many fields such as human health, industries, engineering, electronics, environment and more[2]. Nanoparticles (NPs) have at least one dimension in the range of 1–100 nm[3]. We can obtain nanoparticles from many sources. Some are derived from carbon (Carbon-based NPs) because of their electrical conductivity, great strength, structure, electron affinity and versatility, while others are derived from metals (Metal NPs) i.e. Cu, Ag and Au[4]. The electronic, biomedical, therapeutic and diagnostic properties are the reasons why nanoparticle materials work as catalysts for various kinds of chemical reactions[4]. The effects of many particles have been well documented. However, it is probable that the biological effects of nanoparticles are quite different from that of their microparticles. Nanoparticles show a number of different properties comparative to bulk material[5&6].

Silver nanoparticles (Ag-NPs) are the most popular nanoparticles used as antimicrobial agents[7]. Ag-NPs also have a vast range of biomedical applications[8] such as wound dressing, coatings of surgical instruments and prostheses[9&10]. Their use also extends to food containers and coating material for certain household devices like washing machines. Moreover, they are incorporated into textiles[11&12] and added to cosmetics[13]. Currently, Ag-NPs are being manufactured on

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an industrial scale utilizing physio-chemical techniques like chemical reduction[14], gamma radiation[15], micro emulsion[16], electrochemical methods[17], laser ablation[18], autoclaving[19], microwaving[20] and photochemical reduction[19]. A number of researches have displayed that silver nanoparticles have made their way into therapeutic uses for cancer as anti-cancer agents[21]. Marketable nano-silver is usually obtained as a suspension in pure de-ionized water with nano-sized silver[22].

Despite the fact that a number of recent studies assessed the potential toxicity of Ag-NPs, their action mechanism remains ambiguous. Human exposure to nanoparticles likely happens through inhalation, dermal contact, and ingestion[23]. Owing to the strong affinity of silver for redox-reactive and protective SH groups, the bioavailability of nano-silver in products that consist of Ag-NPs could be enhanced in a physiological environment. Several studies established that the presence of proteins substantially increases the availability of silver from silver dressings[24]. Numerous *in-vitro* studies have proved that silver nanoparticles can enter cells by endocytosis and their location inside the cell may be determined by the perinuclear space of cytoplasm and endolysosomal compartment[25&26].

Ag-NPs are absorbed and distributed in many systems and organs such as the dermis, respiratory, spleen, digestive, urinary, nervous, immune and reproductive system, and largely distributed in the spleen, liver, kidney and lung[27]. The small-sized Ag-NPs are easy to enter the body and penetrate biological barriers like the blood-brain barrier and the blood-testis barrier, and subsequently induce potential cytotoxicity. Besides the directly exposed tissues, Ag-NPs can also be transported to different organs through blood circulation. Therefore, the non-specific distribution of Ag-NPs may produce cytotoxicities such as dermal toxicity, ocular toxicity and respiratory toxicity. The potential cytotoxicity of Ag-NPs depends on the routes of administration and the properties or characteristics of the Ag-NPs, such as the size, shape, and concentration. Further in-depth researches are required to estimate the biocompatibility and potential cytotoxicity of Ag-NPs, which may help to develop harmless and with better biocompatible Ag-NPs-based agents for medical applications[28].

The cytotoxicity of Ag-NPs has been studied on many cancerous cell lines like acute myeloid leukemia (AML) cell lines such as (SHI-1, THP-1, DAMI, NB4, HL-60) and on cells directly isolated from patients[29]. There has been an indication of the toxic effect of Ag-NPs on MCF-7 breast cancer cells by inducing programmed cell death (apoptosis)[30]. Ag-NPs showed a toxic effect on lung cancer cells A549[31]. In addition, by using Ag-NPs promising results were documented in the treatment of human prostate cancer PC3. The researchers concluded that silver nanoparticles exert their cytotoxic effect by inducing apoptosis[32]. Results of other studies showed that Ag-NPs can be used to treat liver cancer cells, where scientists have noticed that Ag-NPs damaged the DNA in this type of cells [33]. When it comes to the effect of these particles on HeLa cervical cancer cells, the emergence of dead crystalline colorless bodies resulting from entering these cells with the programmed cell death pathway after exposing them to different concentrations of nanoparticles has been observed[34].

In the present study, we have highlighted the cytotoxicity of Ag-NPs to human epithelial colorectal adenocarcinoma (Caco-2) cells for a better understanding of the possible actions for such NPs.

MATERIALS AND METHODS

Materials

Silver nanoparticles average diameter: 20 nm +/- 4nm and mass concentration: 0.02 mg/ml, Annexin-V, Propidium Iodide (PI) -purchased from Sigma-Aldrich (USA)-, RPMI (Roswell park memorial institute) medium, fetal bovine serum (FBS), penicillin-streptomycin solution, phosphate buffer saline (PBS) solution, trypsin-EDTA solution -purchased from EuroClone (Italy)-, and 3-[4,5-dimethylthiazol2-yl]-2,5 diphenyltetrazolium bromide (MTT) which was obtained from Genaxxon (Germany).

Cell line

Human colorectal carcinoma cells (Caco-2) were obtained from Atomic energy commission of Syria (AEC). Cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% of 100 u/ml of penicillin and streptomycin. They were grown in a humidified atmosphere of 5% CO_2 and 95% air at 37 C°. Cell culture medium was changed every 2 days and the cells were passaged every 7-8 days.

Cell culture

Cell culture was carried out in Leishmania center, Damascus University. When Caco-2 cells reached the subculturing density of 70-80 % confluence (approximately 9×10^5 cells/cm^2), the medium was removed from the flask and cells were rinsed with 2 mL of phosphate buffer saline (PBS) solution. After that, the flask was incubated with trypsin-EDTA (1x) at 37 C°. Cell suspension was moved into a 15 mL tube and centrifuged for 5 minutes at 1500 rpm. The supernatant was removed, the cell pellet was resuspended in 1 mL of fresh media, and the number of cells was determined by using a hemocytometer after staining the cells using trypan blue dye. Number of cells: the average of cells was calculated in four corner squares $x10^4$ x dilution factor.

Cell viability assay

By using MTT assay, the cytotoxicity of silver nanoparticles was evaluated. This assay is a colorimetric assay for measuring the activity of enzymes that reduce MTT (3-[4, 5-dimethylthiazol2-yl]-2, 5-diphenyltetrazolium bromide, a yellow tetrazole). Viable cells are capable of metabolizing MTT to an insoluble purple formazan crystal. In order to examine the biological effect of Ag-NPs, Caco-2 cells were seeded in a 96-well plate with a density of 1×10^4 cells per well and were grown for 24 hrs. Next, the media was removed and replaced with different concentrations of Ag-NPs 1, 5, 10 and 15 mg/mL. The cells were then incubated with Ag-NPs for 24 and 48 hrs. At the end of the incubation period, 20 µl of MTT (5 mg/ml) were added to each well and the plates were incubated at 37 C° for 3 hrs. After that, 100 µl dimethyl sulfoxide (DMSO) was added at 37 C° for 20 min. to dissolve the purple formazan crystal. At 570 nm, the absorbance was measured using a 96-well plate-reader. The viability of the cell was calculated using the flowing formula:

$$\%\ \text{viable cells} = \frac{abs\ sample - abs\ blank}{abs\ controal - abs\ blank} * 100$$

Calculation of IC50 of Ag-NPS

Linear regression was used to calculate IC50 where the Ag-NPs concentrations were plotted against cell viability in an X-Y figure in GraphPad Prism software, and IC50 was determined using the fitted line: Y= a* X+b

Flow cytometry analysis

In the Atomic Energy Commission of Syria, flow cytometry analysis was performed. Caco-2 cells were plated onto 25-cm^2 flasks and cultured to reach density of 70-80% confluence (approximately 9×10^5 cells/cm^2); incubation with IC50 values of Ag-NPs for 48 hrs. Then Caco-2 treated cells and control cells were harvested, washed twice in PBS and centrifuged 5 min. at 1500 rpm. After removing the supernatant, cell pellet was resuspended in 0.5 mL of 1X Annexin binding buffer, and 5 µL of Annexin-V (50 µg/ml). 2 µL of propidium iodide (100 µg/ml) was added and incubated for 15 minutes in a dark place at 18-25 C°. Finally, cells were analyzed by flow cytometry at an excitation wavelength of 488 nm. The percentages of cells in the G1, S, and G0/G1 phase were analyzed by a computer (Flowjo 7.6.1 software).

Statistical analysis

GraphPad Prism version 8.0 software was used to perform statistical analysis. Student's t-test was utilized to compare two groups and one way. To compare between more than two groups ANOVA was operated. When $p< 0.05$, results were considered statistically significant.

RESULTS AND DISCUSSION

Results

Determination of cytotoxicity of Ag-NPS by the MTT assay

An MTT assay was performed to estimate the cytotoxic effect of Ag-NPs against Caco-2

cell line. Cells were treated with increased concentrations of Ag-NPs for 24 hrs. and 48 hrs. Concentration- and time-dependent effect of Ag-NPs on cells was observed. As shown in figure 1, cell viability of Caco-2 cells at concentration of 1, 5, 10 and 15 µg/mL was 85.7%, 81.1%, 24.8% and 10.9% respectively after incubation for 24 hrs. and 69.5%, 48.8%, 25.45% and 1.7% respectively after incubation for 48 hrs.

Determination of IC50 value of Ag-NPS

To focus on the cytotoxic effect of particular concentration, the half maximal inhibitory concentration (IC50) was calculated as the concentration required to inhibit the growth of tumor cells in culture by 50% compared to the untreated cells. Ag-NPs at 7.85 µg/mL decreased the viability of Caco-2 cells to 50% after 24 hrs. incubation, and this was chosen as the IC50. IC50 value after 48 hrs. incubation was 4.92 µg/mL. Longer exposures resulted in additional toxicity to the cells.

Determination of cell death pathway by flow cytometry

Apoptosis, autophagy and necrosis are the main types of cell death. The Annexin-V analysis by flow cytometry has demonstrated that 11% of the cells Annexin V+ after treatment with 5 µg/mL of Ag-NPs for 48 hrs., while apoptotic cells are about 5% in the control (Figure 3).

Effect of Silver nanoparticles on Cell Cycle Distribution

To determine the effect of Ag-NPs on the cell cycle progression, flow cytometry was used. Ag-NPs treated Caco-2 has shown cell cycle redistribution as demonstrated in Figure 4. The most affected phase of the cell cycle in the treated cell was the G1 phase where it has increased significantly from 49 % in the untreated cells to reach 57 % of treated cells with 5 µg/mL.

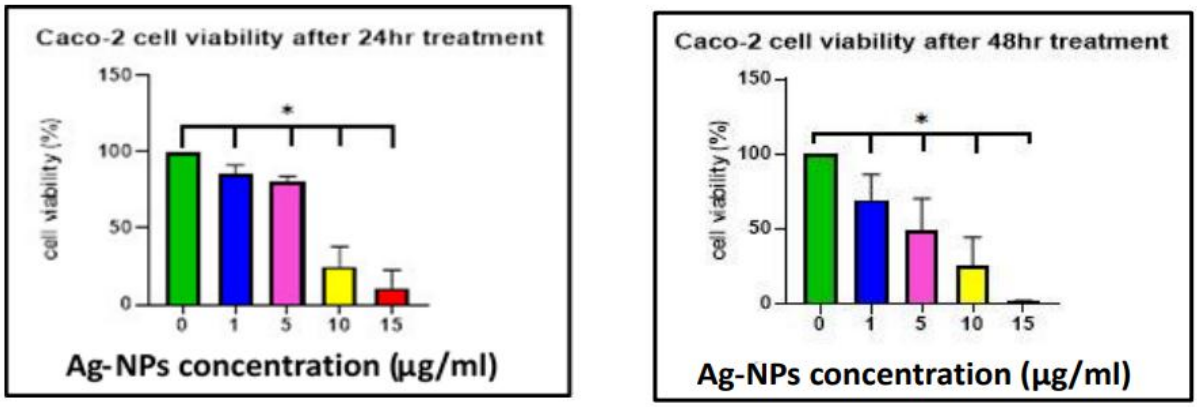

Fig. 1: Effect of Ag-NPs on cell viability of Caco-2 cells. Cells were treated with Ag-NPs at various concentrations for 24 hrs. (A) and 48 hrs. (B), and cytotoxicity was determined by the MTT method. All data are represented as the mean ± SD (n= 2). Treated groups showed statistically significant differences from the control group by the Student's t-test ($p< 0.05$)

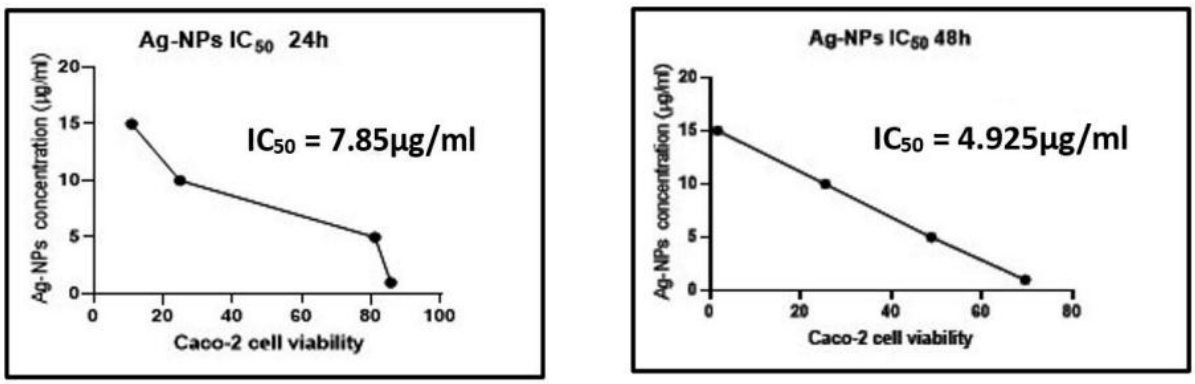

Fig. 2: Cytotoxic activity of Ag-NPs against Caco-2 cell line.

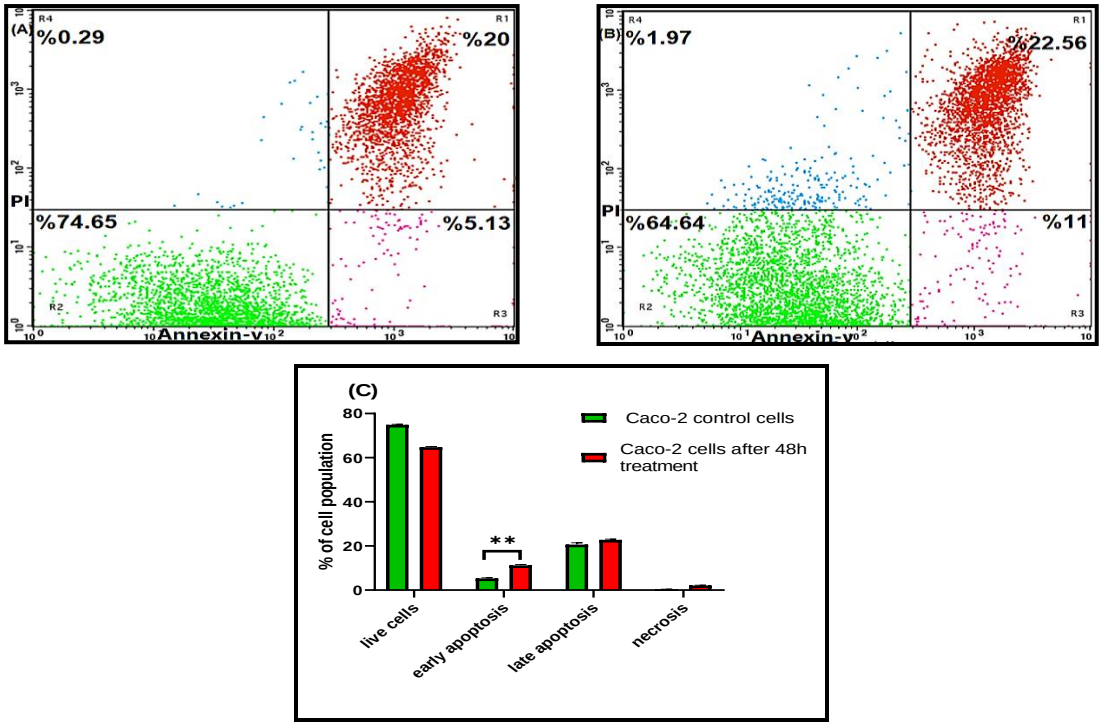

Fig. 3: Apoptosis induced in Caco-2 cells After treatment with 5 µg/mL of Ag-NPs for 48 hrs.Cells were classified as healthy (green color: Annexin V^-/PI^-), early apoptotic cells (Pink color: Annexin V^+/PI^-), late apoptotic cells (red color: Annexin V^+/PI^+), and necrotic cells (blue color: Annexin V^-/PI^+). (A): control, (B) after treatment with 5µg /ml of Ag-NPs for 48 hrs. Annexin-V staining on x-axis and PI on y-axis. (C) Bar diagram describing the percentages of early apoptotic cells, late apoptotic cells, and necrotic cells. Values represent means ± SD of two independent experiments.

The significant change ($p< 0.05$) was the accumulation of the cells in the G1 phase in the treated cells which reflect the increase of subsequently apoptosis.

Discussion

Silver nanoparticles have received attention due to their physical, chemical, and biological properties. Recently Ag-NPs are used as an antimicrobial agent in wound dressings causing reduction in wound inflammation. Developing biocompatible molecule as an anticancer agent is one of the novel approaches in the field of cancer therapy using nanobiotechnology. In the present study, we have used human colon carcinoma cell line (Caco-2) to study the effect of silver nanoparticles Ag-NPs and we have demonstrated that the viability of Caco-2 cells has decreased after treatment with Ag-NPs in concentration- and time-dependent manners. In addition, treatment these cells with Ag-NPs for 24 hrs. or 48 hrs. led to cell death. The toxic pathway seems to be apoptosis rather than necrosis as confirmed by Annexin-V/PI staining and flow cytometry analysis.

Several studies have reported cytotoxic effect of Ag-NPs on various cell lines. Ag-NPs have shown cytotoxicity against human lung cancer cell line A549[29], human cervix HeLa[34], MDA-MB-231 and MCF7[30]. Nowrouzi et al. (2010) observed a concentration-dependent toxic effect of 5–10 nm silver nanoparticles against human hepatoma cell line (HepG2) by XTT and MTT assay[35]. Another study using HepG2 cells treated with 10 nm silver nanoparticles also demonstrated a dose-dependent cytotoxic effect by using MTT and Alamar Blue assay[36]. The results of these studies were in agreement with the results of our study which was designed to assess the potential anticancer activity of the Ag-NPs against Caco-2 cell line. Therefore, an MTT experiment was performed to estimate the cytotoxic effect of Ag-NPs Caco-2 cells.

MTT is an accurate colorimetric method that is commonly used to evaluate cellular toxicity, as well as cell proliferation and viability. Moreover, we found that the cytotoxic properties of Ag-NPs were dose- and time-dependent.

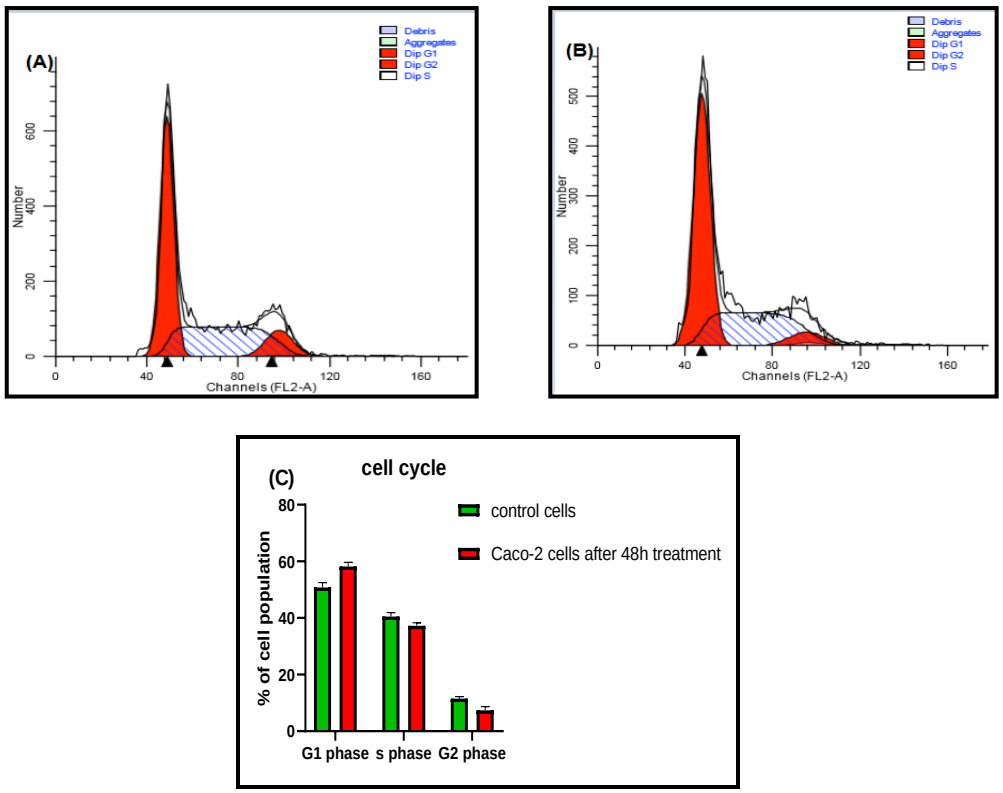

Fig. 4: Flow cytometry analysis of cell cycle phase distribution in Caco-2 cells. Histogram representing propidium iodide (PI) staining of Caco-2 cells without treatment as a control (A) and Ag-NPs (5µg /mL) treated Caco-2 cells for 48 h (B). (C) Bar diagram describing the cell distribution in the G1, S and G2 phases for control cells and treated cells with Ag-NPs (5 µg/mL). Values represent means ± SD of two independent experiments.

Firdhouse et al. (2013) investigated the toxicity of silver nanoparticles on human prostate cancer PC3 and the IC50 value was 6.85 µg/mL. The results suggested that silver nanoparticles exert their cytotoxic effect on prostate cancer cells by inducing programmed cell death (apoptosis)[32]. Sambale *et al.* (2015) investigated the toxic effect of silver nanoparticles on human lung adenocarcinoma epithelial cell line A-549, fibroflasts NIH-3T3, a human hepatocellular carcinoma cell line HEP-G2, and a rat adrenal pheochromocytoma cell line PC-12. In this study Sambale et al. used MTT assay to estimate the cell viability and observed that silver nanoparticles caused a decrease in cell viability in all cell lines and the IC50 values were 4 ppm (µg/mL) for the NIH-3T3 and PC-12 cells, 6 ppm for HEP-G2 and 10 PPM for the A-549 cells[37]. Vila et al. (2017) also established that silver nanoparticles cause a decrease in Caco-2 cells viability and the IC50 value was 12.23 µg/mL after treatment of 24 hrs.[38]. In contrast, the IC50 values in our study were 7.85 µg/ml and 4.92 µg/mL after treatment of 24 and 48 hrs. respectively.

The cytotoxicity of Ag-NPs is influenced by many factors such as concentration, tasted cell lines and particle size[39]. Hussain et al. (2005) investigated the toxic effect of silver nanoparticles with different diameters on rat liver cells BRL3A[40]. The EC50 (Effective concentration 50) value for the silver particles with a size of 100 nm was reported to be 19 ± 5.2 μg/mL, while for particles with a size of 15 nm an EC50 value of 24 ± 7.5 μg/mL. EC50 represent effective concentration of nanoparticles that decreases MTT reduction by 50%[40]. Mirta et al. (2014) investigated the cytotoxic effect of Silver nanoparticles on porcine kidney cells (PK 15), in this study Mirta et al used MTT assay to estimate cell viability, they treated PK 15 cells with various concentration of 405 ± 143 nm Silver nanoparticles for 24h and they found that the decrease in cells viability was 32 % and 54 % after 24 hrs. exposure to 25 µg /ml and 75 µg /ml of Ag-NPs[41], whereas in our study the IC50 value of 20 nm Ag-NPs against Caco-2 cells was 7.85 µg/ml after 24 hrs. of exposure.

Activation of apoptosis pathways was a key mechanism by which cytotoxic silver nanoparticles kill tumor cells, thus, it has became a major goal in cancer therapy. Apoptosis has characterized by cell shrinkage and nuclear DNA fragmentation[42]. Our results of annexin-V/PI assay using flow cytometry has revealed a significant increase of apoptotic cells number after Ag-NPs treatment. Similar result has shown that Ag-NPs induced apoptosis in human cervical cancer cell line HeLa cells within 24 hrs. of treatment[34]. In that study, the emergence of dead crystalline colorless bodies resulting from the entry of the Ag-NPs treated cells into programmed cell death pathway was observed[34]. In fact, the real mechanisms by which the Ag-NPs exerted there cytotoxicity effects is not well understood. Ag-NPs could modify the mitochondrial membrane potential, induce DNA fragmentation, leak lactate dehydrogenase (LDH), activate apoptotic caspases, and generate ROS and nuclear fragmentation[43]. Chen et al. (2016) evaluated and compared the toxicity of three Ag-NPs (20-30 nm with different surface coatings) to Caco-2 after 1-day and 21-days exposures, using various biological assays. In both the short- and long-term exposures, Ag-NPs induced cell growth inhibition and death. The short-term exposure also induced reactive oxygen species (ROS) generation, mitochondrial damage, cell membrane leakage, and apoptosis. The long-term exposure only inhibited the cell proliferation[44].

Carlson et al. worked with 15 nm and 55 nm hydrocarbon-coated Ag-NPs, and found that the 15 nm Ag-NPs can produce more ROS compared with 55 nm Ag-NPs in a macrophage cell line[18]. Liu et al., found that 5 nm Ag-NPs were more toxic than 20 and 50 nm Ag-NPs against four cell lines (A549, HepG2, MCF-7, SGC-7901)[45]. However, Kaba et al. reported that smaller Ag-NPs (particle size was 13.2 ± 4.72 nm) do not play a key role in the viability of tumor cells[46]. The toxic threshold of Ag-NPs was measured as 2 µg/ml for both HeLa and U937 cell lines after 4 h of treatment. The toxic threshold was same for HeLa cells after 24 hrs. of treatment, whereas for the U937 cell line the toxic threshold was 0.05 µg/ml[46]. This might be due to the fact that the interactions of Ag-NPs vary depending on the type of cell types[39].

To summarize, all mentioned studies have resulted in finding a concentration-dependent toxic effect of various silver nanoparticles in numerous cell lines and test methods. Our investigations, in addition to the other cited studies above, clearly show that nanoparticles have many characteristics such as: chemical composition, size, extent, surface chemistry, water and liquid solubility and coagulation or aggregation state, which could be responsible for the toxicity. So far, it remains unclear which properties mostly influence cell cytotoxicity. It is probable that the toxicity of silver nanoparticles may be a combination of various properties, e.g. smaller particles exhibit a greater surface to volume ratio[18]. Nevertheless, the coating, surrounding medium, and state of agglomeration has an influence on the interaction of the nanoparticles with the exposed cells[47].

Conclusion

In this study, the cytotoxicity of Ag-NPs on Caco-2 cells after 24 hrs. and 48 hrs. has been evaluated. It has been concluded that Ag-NPs decrease the viability of Caco-2 cells in a dose- and time-dependent manners. We have also noticed that Ag-NPs are capable of inducing apoptosis in these cells.

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استقصاء الأثر السمي لجسيمات الفضة النانوية على الخط الخلوي Caco-2 الممثل لخلايا سرطان القولون والمستقيم البشري

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تحظى جسيمات الفضة النانوية بأهمية كبيرة نظراً لخصائصها المضادة للميكروبات، فهي تستخدم في الأوعية الحافظة للأطعمة ومواد التجميل فضلاً عن استخدامها في العديد من الأدوات المنزلية. نظرا للاستخدام المتزايد لهذه الجسيمات فقد زادت المخاوف من سميتها المحتملة للإنسان. تهدف هذه الدراسة إلى استقصاء الأثر السّمي لجسيمات الفضة النانوية على خلايا خط خلوي مشتق من سرطان القولون والمستقيم البشري (Caco-2) بالإضافة إلى تحديد مسار الموت الخلوي لتلك الخلايا لأن ذلك قد يساعد لاحقا في معالجة هذا لنوع من السرطانات. تمت معالجة خلايا الخط الخلوي بتراكيز متزايدة من الفضة النانوية ١ و٥ و ١٠ و ١٥ ميكروغرام/مل، مدة ٢٤ أو ٤٨ ساعة. تم بعدها قياس السّمية الخلوية لهذه الجسيمات بواسطة مقايسة MTT .من ثم أجريت تجارب لاختبار الدورة الخلوية ومقايسة الموت الخلوي المبرمج باستخدام مقياس الجريان الخلوي. بينت النتائج أن جميع تراكيز الفضة النانوية سببت انخفاضاً في العيوشية الخلوية لهذه الخلايا بطريقة معتمدة على الجرعة وعلى فترة المعالجة. وكانت تراكيز الفضة النانوية المثبطة لنصف المجتمع الخلوي (IC50) 7.85ميكروغرام/مل و ٤.٩٢٥ ميكروغرام/مل بعد المعالجة لمدة ٢٤ ساعة و ٤٨ ساعة على التوالي. كما أظهرت نتائج اختبار كلاً من الدورة الخلوية والموت الخلوي المبرمج، زيادة معنوية في النسبة المئوية للخلايا المتراكمة في الطور G1 من الدورة الخلوية، وزيادة معدل الموت الخلوي المبرمج بنسبة ٦٪ وذلك في الخلايا المعالجة بالتركيز ٥ ميكروغرام/مل من الفضة النانوية لمدة ٤٨ ساعة. وبالخلاصة توصلت الدراسة إلى أن جسيمات الفضة النانوية تخفض عيوشية خلايا سرطان القولون والمستقيم بطريقة معتمدة على الجرعة وعلى الزمن. كما لوحظ أن هذه الجسيمات قادرة على تحريض هذه الخلايا على التوقف عن الاستمرار في الدورة الخلوية وعلى الدخول في الموت الخلوي المبرمج.