Gemcitabine and Synthesized Silver Nanoparticles Impact on Chemically Induced Hepatocellular Carcinoma in Male Rats

Soheir A. Osman
National Centre for Radiation Research and Technology, Egyptian Atomic Energy Authority

Shadia A. Fathy
Ain Shams University

Mohamed R. Mohamed
Ain Shams University

Amany I. Raafat
National Centre for Radiation Research and Technology, Egyptian Atomic Energy Authority

Mahmoud M. Refaat
National Centre for Radiation Research and Technology, Egyptian Atomic Energy Authority

Asmaa A. Hassan (asmaabubakr72@gmail.com)
National Centre for Radiation Research and Technology, Egyptian Atomic Energy Authority

Research Article

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Abstract

Purpose

Gemcitabine (GEM) is a deoxycytidine analog chemotherapeutic drug widely used for the treatment of many cancers. Silver nanoparticles (AgNPs) are important nanomaterials used in the treatment of many diseases such as infectious diseases. Using gamma radiation in nanoparticle preparation is a new eco-friendly method. This study aims to evaluate the efficiency of the co-treatment of gemcitabine and silver nanoparticles in hepatocellular carcinoma treatment.

Methods

AgNPs characterization has been done using UV-visible spectroscopy, XRD, TEM, and EDX. Firstly, the MTT cytotoxicity in vitro assay of gemcitabine, doxorubicin, and cyclophosphamide was assessed against Wi38 normal fibroblast and HepG2 HCC cell lines. Then after HCC development, rats received (10µg/g b.wt.) of AgNPs three times a week for four weeks and/or GEM (5mg/kg b.wt.) twice weekly for four weeks. Liver function enzymes were investigated. Cytochrome P450 and miR-21 genes were studied. Apoptosis was determined by using flow cytometry, and apoptotic modifications in signaling pathways were evaluated via Bcl-2, Bax, Caspase-9, and SMAD-4.

Results

The co-treatment of GEM and AgNPs showed upregulation of apoptosis by increasing Bax and caspase 9 while diminishing Bcl2 and SMAD4, amelioration of cytochrome P450 m-RNA relative expression. In addition, the results proved the cooperative effect of GEM and AgNPs in the deactivation of miR-21.

Conclusion

The impact of AgNPs as an adjuvant treatment with GEM was recognized. The interaction between AgNPs and Gem can diminish some of the drawbacks of using GEM alone and elevate its efficiency in HCC treatment via enhancing intrinsic and extrinsic apoptotic pathways.

Introduction

Liver cancer, an asymptomatic disease, is the fourth most common type of cancer and the second most common cause of cancer-related mortality globally (Singh et al, 2018, Yang et al, 2019). Hepatocellular carcinoma (HCC) is one of the most common primary liver cancers (Massalha, et al., 2020), it is considered the 3rd cause of mortality worldwide, and it is documented as the last step for chronic liver diseases and is firmly related to fibrosis and cirrhosis (Moreira et al 2015). Additionally, HCC occurrence is accompanied by inflammation and oxidative stress (Wang et al., 2016). Considering the lack of effective treatment options and the poor prognosis of surgical intervention, chemoprevention remains the best strategy to combat HCC (Reddy et al., 2009). Nanoparticle-mediated targeted therapy is an advanced and promising alternative that may be more effective than conservative chemotherapeutics (Yuan et al.,
2017), because of their unique properties (Chung et al., 2016). When materials reach Nano-size, they exhibit a higher surface area to volume ratio, which makes them potentially more reactive (Miresmaeili et al. 2013). Silver nanoparticles (AgNPs) provided a novel approach to overcoming tumors, especially those of hepatic carcinoma (Zhu et al., 2016). Furthermore, silver nanoparticles exhibit low toxicity in humans and have diverse in vitro and in vivo applications (Vasanth et al., 2014). Thus, exposure to silver nanoparticles as a promising anticancer adjuvant treatment is becoming increasingly intimate and widespread. Gamma irradiation (γ-irradiation) is a powerful technique to synthesize nanoparticles (NPs) of controlled size and shape (Jurkin et al., 2016). It proved to be a simple and efficient method for silver nanoparticle synthesis (El-Batal et al., 2013). Gemcitabine (GEM; 2’-deoxy-2’, 2’-difluorocytidine) is a nucleoside analog used widely as a chemotherapeutic agent for many cancers; ovarian, bladder, non-small cell lung, pancreatic, and breast carcinoma (Qi et al., 2016). It is a promising therapeutic candidate for treating HCC (Devulapally and Paulmurugan, 2014). Using combinations of nanoparticles and classical chemotherapeutic drugs may provide better curative effects than does the single line of treatment. Actually, there is no documents on the use of the combination of GEM and AgNPs in the treatment of HCC. To accomplish the effectiveness of the combination, AgNPs synthesized by γ-irradiation then characterized. MTT cytotoxicity done for AgNPs and GEM on HCC and normal cell lines. At last the potential of this combination in induction of apoptosis was performed.

Materials and methods

Materials

Chemicals:

Gemcitabine [C9H11F2N3O4], obtained from Eli Lilly Company. Indianapolis, Indiana 46285 USA. Silver nitrate (MW 169.87), PVP (MW≈ 40,000), isopropyl alcohol, and diethyl-nitrosamine (DEN) were acquired from Sigma-Aldrich Corporation (USA). Normal human fibroblast cells (Wi-38) and human liver cancer cells (HepG2) were obtained from the National Research Center, (the tissue culture unit of the Holding Company for Biological Products and Vaccines (VACSERA), Giza, Egypt.

Gamma Irradiation source:

Irradiation was performed at NCRRT, EAEA, Cairo; within a γ-irradiation system using Canadian Gamma cell-40 (60Co source) as a reducing source, with a dose rate of 1.06 kGy/h at irradiation dose 25 kGy.

Animals:

Fifty-six male albino rats weighing 110 - 130 g, gained from the National Center for Radiation Research and Technology (NCCRRT) were housed in an environmentally controlled clean-air room with standard temperature and maintained a 12 h light/ dark cycle. They were kept on standard food pellets containing all nutritive elements and liberal water ad libitum, as per (Clarke et al., 1977) and (MS et al., 2004). All
animal procedures are carried out in compliance with the guidelines of the NCCRT and with guidance for the proper treatment and use of laboratory animals.

**Synthesis and Characterization of the silver nanoparticles (AgNPs)**

Silver nanoparticles were manufactured using $^{60}$Co-gamma irradiation as a clean tool for synthesis. Silver nitrate (AgNO$_3$) was used as an Ag$^+$ ions precursor, PVP as a stabilizer, and 2-propanol as a scavenging oxidizing agent. Typically, (5ml) of 0.1 M AgNO$_3$ was added to 50 ml of 1% PVP solution and 0.5 ml isopropanol then the mixture was stirred well. After being purged by N$_2$ for 20 min, the mixture was sealed and exposed to a 25 kGy dose using gamma rays at room temperature. Following irradiation, Ag$_0$NPs were separated by centrifugation, to remove the free PVP and excess silver ions. The precipitated Ag$_0$NPs were thoroughly washed with ethanol, dried at room temperature then stored for further investigations. X-ray diffraction (XRD) patterns of the obtained Ag$_0$NPs were studied using XRD 6000 diffractometer with a Cu target. The XRD runs were carried out over 2$\theta$ ranging from 10° to 80° at a scan speed of 8°/min. The UV-visible wavelength scan for the absorption spectra was obtained using a UV/Vis spectrometer model UV-Analytic Jena AG specord 210 plus (Germany) at a wavelength of (190–900) nm. EDX analysis was performed using energy dispersive X-ray unit microprobe EDX (Oxford, England - ISIS). The size of AgoNPs was estimated using transmission electron microscopy (TEM) (JEOL, JEM 100CX, Japan). The obtained Ag$_0$NPs were dispersed in methanol followed by sonication then a drop of the obtained solution was dropped on an ultrathin carbon-supported Cu grid and air-dried at room temperature.

**Methods**

**MTT Cytotoxicity assay:**

Cytotoxicity assay was performed on both Wi-38 and HepG2 cell lines; carried out according to (van de Loosdrecht et al, 1994). MTT reagent [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] was used. In brief, in 100 µl of phosphate-buffered saline (PBS, 0.2 M, PH=7.4), the (2.5x 10$^6$) cells were seeded and mixed with 100 µl of various concentrations of drugs; Gemcitabine (GEM) Doxorubicin (Doxo), and Cyclophosphamide (Cyclo) (1000 - 7.81 µg/mL) in a 96-well -plate and incubated for 24 hrs. at 37°C,10%CO$_2$. HepG2 and Wi38 cells in PBS without treatment were considered as control (100% viable). 10 µl from (0.5 mg/ml) MTT freshly prepared solution was added to each well. The plate was incubated for 4hrs in the dark; to develop color. 200 µl of DMSO/well was added to dissolve MTT formazan, and the absorbance was measured at 570 nm. The percentage of cell cytotoxicity was calculated as follows:

Viability % = sample abs /control abs x 100 and Cytotoxicity % =viability % -100

**Experimental design:**
The rats were randomly categorized into 8 groups (n=7) as follows: (1) Control group (C); Normal male rats were injected intraperitoneally (i.p.) with 0.9% saline solution. (2) Silver nanoparticles group (Ag); animals were i.p. injected with AgNPs (10 μg/g) /b.wt 3 times a week, for 4 weeks (He et al., 2016) (Kadhim, et al 2017) with some modifications. (3) Gemcitabine (G); rats of this group were injected (i.p.) with GEM 5 mg/kg twice weekly, for 4 weeks (Liu et al, 2015) with modifications. (4) Rats treated with AgNPs and GEM (AgG); rats of this group were injected in groups 2 and 3. (5) HCC group (T); rats were orally administrated with 20 mg/kg b.wt. of DEN (dissolved in 0.9% normal saline), five times a week for eight weeks, followed by 10 mg/kg for another two successive weeks conferring to the modified method (Karimov et al, 2003) and (Darwish and El-Boghdady, 2013). (6) HCC-AgNPs treated rat group (TAg); rats were injected as group 5 and then treated as group 2. (7) HCC-GEM treated rat group (TG); Rats developed HCC as in group 5 and treated as group 3. (8) HCC-AgNPs-G treated rat group (TAgG); Rats advanced HCC, as in group 5, treated as group 4.

Samples collection and preparation

Blood was drawn from the heart and separated into two parts; whole EDTA blood for flow cytometry analysis and serum for other biochemical investigations. Liver tissues were excised and dissected for assessment of RT-qPCR and western blotting analysis. The other part was washed and kept in 10% formalin for histopathological studies.

Flow cytometry analysis.

Apoptosis was measured as a FITC Annexin V Apoptosis Detection Kit I (Cat. No: 556547), according to (Casciola-Rosen, et al 1996). Whole blood samples from EDTA collection tubes were directly processed according to Yakimov et al., 2019

Quantitative real-time PCR:

The sequence of the PCR primer pair used for miR-21 and cytochrome c are shown in Table 1. Data were studied with the ABI Prism sequence detection system software and then quantified using the v1·7 Sequence Detection Software from PE Biosystems (Foster City, CA). Relative expression of the studied gene was measured using the comparative threshold cycle method. All values were normalized to the endogenous control Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) (Livak and Schmittgen 2001).

Table (1): Sequence of primers for the selected genes
Western blot analysis

The protein expression of BCL2 (Cat. No: ab194583 ~ 26kDa), Bax (Cat. No: ab182733 ~ 21 kDa), SMAD4 (Cat. No: AF5247 ~ 65 kDa), and Caspase-9(Cat. No: AF6348 ~ 46 kDa) was estimated in liver tissue. Data of the obtained protein levels were estimated using β-actin as a housekeeping arbitrary unit (A.U) β-actin (Cat. No: ab8227 ~ 42 kDa).

Histopathological investigations.

Specimen from liver tissue of all studied groups was washed, dehydrated in ascending grades of ethyl alcohol, cleared in xylene, and embedded in paraffin wax. Sections of 5–6 mm in thickness were cut out, deparaffinized, and stained with Hematoxylin and Eosin (H & E) for examination under the light microscope (Banchrof et al., 1996).

Statistical analysis:

Statistical analysis of results was executed via SPSS version 20.0. All data are given as means ± standard error (SE). Differences were considered significant at p<0.05, and Data were analysed statistically using one-way ANOVA followed by Tukey as Post Hoc analysis.

Results

Radiation synthesis of silver nanoparticles Ago NPs

The irradiation preparation method has many advantages; energy and materials saving and environmentally friendly. When Ag⁺ aqueous solution is subjected to gamma irradiation, the predominant initiating chemical step is the water molecules radiolysis accompanied by the formation of hydrated electrons ($e_{aq}^−$) and primary radicals and molecules such as; hydroxyl radicals (OH), hydrogen atoms (H⁺), H3O⁺ ions, and molecules; dihydrogen H₂ and hydrogen peroxide H₂O₂. The solvated electrons ($e_{aq}^−$) and H⁺ atoms are strong reducing agents. In order to enhance the reducing conditions, 2-propanol was used to
scavenge hydroxyl (’OH) radicals. ’OH, radicals are able to oxidize ions or atoms into a higher oxidation state, and thus compensate for the reduction reactions. In addition to hydrated electrons (e\textsubscript{aq}\textsuperscript−) the 2-propanol radicals (CH\textsubscript{3})\textsubscript{2}C’OH are also reducing in nature. These reducing radicals will reduce Ag\textsuperscript{+} ions to Ag atoms which subsequently form AgNPs. The formation of Ag\textsuperscript{0}NPs from aqueous solutions of AgNO\textsubscript{3} stabilized PVP in the presence of isopropanol using gamma irradiation involves the following reactions (Stamplecoskie & Scaiano,2010 Nasseri et al 2019)

\[
\begin{align*}
H_2O \rightarrow & \quad e\textsubscript{aq}^- + H_3O^+ + H^+ + OH^+ + H_2 + H_2O_2 + \ldots \\
Ag^+ + e\textsubscript{aq}^- \rightarrow & \quad Ag^0 \\
Ag^+ + H^+ \rightarrow & \quad Ag^0 + H^+ \\
Ag^+ + (CH_3)\textsubscript{2}C’OH \rightarrow & \quad Ag^0 + (CH_3)\textsubscript{2}CO + H^+ \\
Ag^0 + Ag^+ \rightarrow & \quad Ag_2^+ \\
Ag_{n-1} + Ag^+ \rightarrow & \quad Ag_n^+ 
\end{align*}
\]

Characterization of the radiation-synthesized silver nanoparticles

- UV−vis spectroscopy analysis

Ag\textsuperscript{0}NPs are readily detectable by UV-Vis spectroscopy because of their characteristic absorption maximum ranging between 380–430 nm. Figure (1a) shows the UV/V absorption spectrum of the obtained colloidal Ag\textsuperscript{0}NPs. As clear, a characteristic absorption band of metallic Ag\textsuperscript{0}NPs surface plasmon resonance (SPR) absorption band appears at \(\lambda_{\text{max}}\) (420 nm) which is compatible with the reported spherical AgNPs absorption band (Raafat et al 2018). Interestingly, Ag\textsuperscript{+}NPs have a conduction band and a valence band that is very close to each other, allowing electrons to move freely between them. Owing to the collective oscillation of electrons of silver nanoparticles in resonance with the light wave, the characteristic surface plasmon resonance (SPR) absorption occurs (Rodriguez et al, 2020).

X-ray diffraction (XRD) analysis

As clear from Fig. (2a), the XRD spectrum of the obtained Ag\textsuperscript{0}NPs shows well-defined characteristic diffraction peaks at 2\(\theta\) values at 37.9°, 44.26°, 64.50°, and 77.29 assigned to diffractions planes (111), (200), (220), and (311), respectively confirming the formation of face centered cubic (fcc) structure of Ag\textsuperscript{0}NPs. The average crystallite size of the developed Ag\textsuperscript{0}NPs was calculated from XRD pattern using Scherrer’s equation (Raafat et al 2020):

\[
D = \frac{k\lambda}{\beta\cos\theta}
\]
where D is the average crystallite size, $\beta$ is the full width of the peak at half of the maximum intensity (rad), $\lambda$ is the wavelength of the monochromatic X-ray beam ($\lambda = 1.5406\text{Å}$), $\theta$ is the peak diffraction angle (Bragg’s angle), and K is a Scherrer constant defined as the crystallite shape and approximately equals 39 nm.

**Transmission Electron Microscopy (TEM)**

As clear from Fig. (2b), the TEM image confirmed the nanoscale size of the obtained Ag$^0$NPs. The Ag$^0$NPs were found to be spherical and a relatively narrow size distribution with an approximate average size ranged between 30–50 nm which correlates with the XRD measurements.

In addition, The XRD spectrum and TEM image confirmed the nanoscale size of the obtained Ag$^0$ nanoparticles, which was in good agreement with the particle sizes calculated according to Scherrer’s equation. Finally, the successful preparation was confirmed by EDX analysis which showed the sharp signal peak of silver powerfully established the reduction of silver nitrate to Ag-NPs.

**-Energy dispersive X-ray spectroscopy (EDX)**

As clear from Fig. (3), the appearance of a strong signal for silver atoms in the range of 3keV, which is a typical signal of the absorption of metallic and spherical silver nanocrystals due to surface plasmon resonance, confirmed the elemental composition of the prepared Ag$^0$NPs according to the EDX analysis profile and attested to the success of the Ag$^0$NPs preparation (Akshay et al 2018).

**MTT cytotoxicity assay**

The mitochondrial activity of Wi38 normal fibroblast cells and HepG2 hepatocellular carcinoma cell lines were evaluated by an in vitro MTT assay. Wi38 and HepG2 cells in PBS were considered as the control (100% viability and 0% toxicity). The drug concentrations used were (7.8–1000µg/ml). First, the cytotoxicity of all drugs was estimated on the normal cell line Fig. (4) which revealed that doxorubicin showed the highest cytotoxic effect on Wi38 cells (48.7%) and cyclophosphamide (30.6%) where gemcitabine recorded (34.65%). More interestingly, cyclophosphamide has a lesser cytotoxic effect on the HepG2 cells Oppositely, doxorubicin is the most cytotoxic drug among them but it is also the most cytotoxic drug to the normal cells. IC50 was assessed for the three drugs (Gemcitabine 44.5, Doxorubicin 6.45, and Cyclophosphamide 190.56 µg/ml) (Fig. 5).

**Liver function enzymes examination**

The liver function enzyme activities were examined in all tested groups. There were significant elevations ($P < 0.05$) in the HCC (T) group compared to their equivalent values in the control. Meanwhile, the treatment of Gem showed a potent decrease compared to the T group. However, associated AgNPs treatment showed a significant amelioration ($P < 0.05$) in their activities in the TAg group compared to the T group. There was a synergistic impact between Gem and AgNPs appeared in all enzyme activities.

**Annexin V Apoptosis analysis**
Apoptotic Annexin V/PI lymphocyte distribution using flow cytometry analysis was shown in dot plots and histograms (Fig. 7&8). Annexin V/PI staining defined the apoptotic and non-apoptotic population of cells. In the present work, the total apoptotic activity (V+/PI-) and (V+/PI+) significantly increased in T group (63.6 ± 0.32) comparing to the healthy control group (0.15 ± .08). However, our results demonstrated that Gem can reduce the total apoptosis of lymphocyte (50.07 ± compared to T group. In addition, the AgNPs administration showed a potent decrease in apoptosis (43.5 ± 0.46) compared to T group. Remarkably, the combination of AgNPs and Gem established a synergistic effect counter HCC development in TAgG (35.68 ± 0.58) contrary to the T group.

**Mixed function cytochrome P-450 enzyme activity**

In the present work, the expression of liver CYT P450 mRNA of the control(C) group was 1.06 ± 0.09 folds. The developed HCC group (T) exhibited a significant increase in CYT P450 mRNA compared to the control group. Nevertheless, the results revealed significant improvement in the HCC group treated with AgNPs (TAg) (5.31 ± 0.38) or Gem (TG) (4.51 ± 0.43) compared to the T group (9.20 ± 0.47) (P < 0.05). Moreover, the combined treatment of AgNPs and Gem (TAgG) observed a potent improvement (2.80 ± 0.22) compared to the T group (P < 0.05). (Fig. 9)

**Apoptotic pathway detections**

The protein expression of Bax was significantly decreased (P < 0.05) in the T group (68.46%) compared to the control group (Fig. 10a). On the other hand, the treatment by AgNPs alone (TAg), GEM alone (TG), or both (TAgG) significantly elevated (P < 0.05) (7.07-fold change,9.5-fold change, and 14.56-fold change, respectively) compared to the T group. Similarly, Caspase9 protein expression demonstrated a significant decline (P < 0.05) in the T group (64.47%) compared to the corresponding control. However, the protein expression of Caspase9 significantly rise (P < 0.05) (7.5-fold change,11.08-fold change, and 16.25-fold change, respectively) compared to the T group (Fig. 10b). Though, the protein expression of BCl2 exhibited a potent increase in T group (P < 0.05) (5.5-fold change) in comparison to the respected control. Conversely, the data of the TAg, TG, and TAgG displayed a significant reduction (p < 0.05) (35.42%,43.82%, and 63.59%, respectively) corresponding to tumor developed group (T) (Fig. 10c).

The protein expression of SMAD4 is presented in Figure (11). The expression of the T group was increased significantly (P < 0.05) (5.4-fold change) compared control group (C). Otherwise, the treatment by AgNPs or Gem alone or together TAgG demonstrated a significant decrease (P < 0.05) (34%, 45%, and 56.68, respectively) compared to the T group.

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The gene expression of miR-21 in the T was significantly elevated (P < 0.05) (8.40-fold change) compared to the control. Oppositely, miR-21 expression in TAg, TG, and TAgG groups was potently declined (32.36%,39.2%, and 62.25%, respectively compared to the T group (Fig. 12)

**Histopathological study.**
The normal histological structure of the hepatic tissue is shown in Figure (13). Remarkably, in the control group there is no pathological clues were shown. In rats treated with ANPs, there was no toxicity according to AgNPs treatment that the normal-sized portal tract surrounded by cords of average-sized hepatocytes with no degeneration or necrosis was presented (Fig. 13b). The liver section of the G group is presented in Figure (13c) showed central veins enclosed by cords of degenerated hepatocytes, some of them showed patchy necrosis. The combined administration of AgNPs and Gem in the AgG group showed a minor dilated central vein surrounded by cords of slightly degenerated hepatocytes, some showing pyknotic (dark stained small) nuclei and apoptotic bodies (Fig. 13d). Nevertheless, rats that developed HCC revealed sheets of malignant hepatocytes exhibiting pleomorphism, hyperchromasia, abnormal mitotic figures, and focal areas of necrosis (Fig. 13e). Additionally, Figure (13f) presented the liver section of the TAg group showed malignant cells surrounded by 80% of tumor necrosis. In TG group the section of liver showed malignant cells surrounded by 60% of tumor necrosis (Fig. 13g). But rats treated with AgNPs and Gem showed few residual malignant cells surrounded by 95% of tumor necrosis, illustrated in Figure (13h)

Discussion

Owing to the unique characteristics of nanoparticles, they may be one of the promising modalities for cancer treatment with traditional chemotherapeutic drugs. Gamma radiation is an eco-friendly method for silver nanoparticle synthesis, compromising plentiful advantages over conventional chemical and photochemical procedures because of its simplicity and efficiency (Yoksan and Chirachanchai 2009). The radiochemical process was achieved to reduce Ag + ions at ambient temperature without using excessive reducing agents or producing unwanted by-products of the reductant. In addition, the reducing agents were distributed uniformly in the solution, so AgNPs were formed in highly pure and stable forms (Sheikh et al, 2009). The size of nanoparticles is considered a vital factor in their cytotoxicity and cellular uptake (Fehaid and Taniguchi, 2019). It affects their interaction with mammalian cells and where NPs accumulated (Sriram et al, 2012). In the current work, AgNPs exhibited a diameter of 30–50 nm. Many reports documented that decreasing the size leads to an increase in the surface area of the particles, which facilitates the diffusion of the particles into the cell (Abou El-Nour et al, 2010). The polymeric materials coating AgNPs enhance their stability and reduces the Ag + ions dissolution from AgNPs surface. (Mishra et al, 2016). Using different coating materials can affect AgNPs toxicity; Anderson et al, 2015 documented that PVP-coated AgNPs directed to the lung get cleared faster than citrate-coated AgNPs, so surface coating has a vital role in the internal clearance and translocation of AgNPs. Therefore, we prepared PVP-coated AgNPs to sidestep any coating-dependent disparity in cellular response (Peloquin et al 2020) (Priya et al 2020). Fehaid et al 2020 recommended PVP for AgNPs coating in vitro studies because of their higher stability with less aggregation and dissolution changes. The efficiency of AgNPs on HCC-induced rats was studied by measuring liver function enzyme (ALT, AST, and ALP) activities; they are indicators of hepatocellular injury. In healthy (control) liver, organ homeostasis happens through cell death and tissue regeneration. Therefore, ALT and AST levels were maintained within a normal range (Katoonizadeh 2017). However, a significant increase in liver enzyme activities was
documented regarding HCC development due to hepatocyte injuries, so enzymes release into the blood circulation (Taha et al., 2021). ALT is a more specific biomarker for acute liver injury at its early stages and in some types of cancers, such as HCC (Luedde et al., 2014). But both enzymes’ activities were decreased after AgNPs treatment (TAg) which may confirm the AgNPs role in the probable regeneration of hepatocytes and possible healing of the hepatic parenchyma (Zhang et al., 2019). The comparative cytotoxic study was carried out by MTT assay to evaluate the cytotoxic effect of gemcitabine, doxorubicin, and cyclophosphamide drugs on Wi38 and HepG2 cell lines. The results documented the dose-dependent inhibitory ability of GEM against HepG2 cells more than cyclophosphamide and less toxic on the normal Wi38 cells. Apoptosis is the first guard against cancer progression (Wong, 2011). Furthermore, much research defined the disturbance of apoptotic mechanisms during cancer development (Kashyap et al., 2021). Annexin-V results showed a value of (63.6 ± 0.32) in the lymphocytes of HCC-developed rats (T) due to the capability of cancer cells to alter the levels of proteins associated with lymphocyte function, including apoptosis (Bin-Alee et al., 2020). The amount of apoptosis in the T group was approximately 1.5 times of rats treated with AgNPs (TAg) (43.5 ± 0.46). Farahani et al., 2020 stated the ability of AgNPs to induce apoptosis in cancerous cell lines with low toxicity on normal liver cells and the lymphocyte. Mahjoub et al., 2022 showed that AgNPs administration did not cause changes in the WBC formula, but it can cause activation in murine lymphocytes after a few hours of exposure to AgNPs. In addition, in the group treated with GEM, the apoptosis was markedly decreased (50.07 ± 0.32) regarding the T group. Furthermore, the combination of AgNPs and GEM exhibited a more pronounced effect on lymphocyte apoptosis (35.68 ± 0.58) as the co-treatment improves therapeutic efficiency and eliminates undesired side effects. Besides, our investigation exhibits the mechanism understood that AgNPs mediated extrinsic and intrinsic apoptotic cell death. The proteins of apoptotic factors Bax, Caspase9, and the anti-apoptotic Bcl2 were isolated, and their expression levels were determined, that illustrated in Fig. (10). The displayed results revealed that Bcl-2 expression was suppressed and Bax expression was up-regulated in rats treated with AgNPs compared to the control group. It has been proven that the boosted expression of Bax represents the main effector of the intrinsic pathway responsible for the induction of mitochondrial modifications that leads to cytochrome c and caspase – 9 induction (Jendrossek, 2013). Kanipandian et al., 2019 proved that the decreased Bcl2 level indicates that AgNPs effectively interfere with the intrinsic apoptotic activity by preventing the anti-apoptotic gene expression. Remarkably, it was demonstrated that the up-regulation of caspase 9 provokes caspase – 3 levels in AgNPs treated cells (Kanipandian et al., 2019). Ahmadian et al., 2018 demonstrated that AgNPs induce Bax gene expression and decrease Bcl2 expression. Rakowski et al., 2021, reported that depending on the size surface modification, and shape of AgNPs can induce cytotoxicity, mitochondrial dysfunction, and oxidative stress also enhance apoptosis. Moreover, in this study, GEM enhanced apoptosis by up-regulation of Bax and caspase9 and downregulation of Bcl2 protein expression; this data is compatible with Yuan et al., 2017 documented that GEM single treatment can induce DNA fragmentation, also Teng et al., 2018 documented that GEM not only inhibits cell proliferation and angiogenesis in tumor cells but also induces apoptosis. Zhu et al., 2018 verified that GEM induces apoptosis in pancreatic cell lines by inhibiting Bcl2. Moreover, many previous studies demonstrate that GEM induced apoptosis by improving Bax and decreasing Bcl2 expression in breast cancer cells (Cao et al., 2019) and gall bladder cancer cells.
(Ruan et al, 2021). Further, cytochrome P450 (CYP) is an enzyme family that plays vital roles in the endogenous and exogenous metabolism of many molecules (Zhou et al, 2016). They are the main enzymes incorporated in cancer development. The present results showed that AgNPs effectively decrease the elevation of CYP2E1 that increased in the HCC group activated by DEN. It was reported that CYP enzymes mediate the metabolic activation of many procarcinogens (Zhou et al, 2016). They can hydrolyze diethylnitrroseamine in the liver into active metabolites (Kang et al, 2007). Kang et al, 2007 proved that CYP2E1 is the key diethylnitrroseamine-activating P450 responsible for diethylnitrroseamine-induced hepatocarcinogenesis. Also, Munger et al, 2015 stated the impact of nanoparticle size on cytochrome P450 enzymes. The average size of AgNPs in this study is 30-50nm, which verifies the data presented by Fröhlich et al., 2010 who documented that small (20–60 nm diameter) non-metallic, carboxy polystyrene particles inhibited the enzymatic activity of some CYP subtypes in normal liver microsomes. In addition, the treatment with AgNPs and GEM showed a potent amelioration in CYP2E1 corresponding to the tumor group. Munger et al (2015) displayed that CYP2E1 had intermediate inhibition caused by nano-silver exposure. Additionally, Li et al, 2017 showed that overexpression of CYP2E1 induced cytotoxicity to HepG2 cell line. Further, miR-21 was studied in this work; microRNAs (miRNAs) are small noncoding RNAs of about 22 nucleotides (Qu et al, 2019); they are participated in the post-transcriptional regulation mechanisms (Wang et al, 2019) in a wide variety of cellular functions, including apoptosis, cellular proliferation, DNA damage response, neovascularization, immunological response, stress response, and most vital is tumor progression (Rhim et al, 2022). miR-21, is an onco-miR frequently overexpressed in various cancer types (Gheytanchi et al, 2023). The gained results revealed a significant elevation in miR-21 expression in tumor progressed group(T), that consistent with Pu et al, 2017 who proved that miR-21 is related to HCC and considered as HCC serum biomarker. Xu et al, 2013 stated that, miR-21 participated in the carcinogenic process by targeting many vital signaling processes; apoptosis, cell proliferation, and invasion. It has been found previously that miR-21 is elevated in patients with hepatitis by downregulation of SMAD7, a negative regulator of (TGF)-β signaling which in turn induces miR-21 (Oura et al, 2020). Remarkably, Buscaglia and Li (2011) reported that miR-21 inhibits apoptosis by downregulating Bax and upregulating Bcl2. Also, they postulated that, throughout hypoxia, miR21 is downregulated and FasL is activated due to lack of targeting of the FasL by miR21. Oppositly, during cancer growth, miR21 is activated which decreases FasL and its proapoptotic signaling. The down-regulation of miR21 can cause apoptosis via increased amounts of caspases 9 and 3. So, using AgNPs alone or in addition to GEM showed a significant diminish in miR-21 expression through the enhancement of caspase9 and Bax and Bcl2 downregulation(Fig. 10).

SMAD4 is a transcription factor of the SMAD family which regulates TGF-β signal transduction (Lin et al, 2019). The TGF-β/SMAD signaling pathway is one of the most frequently transformed cellular pathways in human cancers and plays a twin role in tumorigenesis (Wang et al, 2021). Depending on the tumor stage, TGF-β/Smad4 signaling pathway works; in the early stage, it exhibits a suppressive role by inducing apoptosis, while later on in the late stage, TGF-β/Smad4 boosts tumor immunosuppression and simplifies tumor angiogenesis, invasion, and metastasis (Zhao et al, 2018). In this work, SMAD4 protein expression proves the role of SMAD4 in HCC progression, its protein expression was significantly elevated
in the HCC group (T) compared to the control. This data was confirmed by Yao et al 2012 who stated that Smad4 protein is over-expressed in HCC and is correlated to poor prognosis. Also, Hernanda et al 2015 revealed a significant increase in nuclear SMAD4 localization in patient HCC tumors, which attached to tumor-promoting effects because of the simultaneous rise of p-SMAD2/3 in the subset. HCC is classified as an inflammation-related tumor (Hiwatashi et al 2009) and showed a potent increase in TGF- β level that was controlled by Smad4 elevation; these results were published in our previous work (Refaat et al, 2023). Moreover, many previous studies documented the role of Smad4 and mir-21 in HCC-drug resistance, especially GEM resistance (Liu et al 2016). So, by AgNPs administration as co-treatment with GEM, Smad4 expression was significantly decreased (TAgG).

**Conclusion**

In the current work, we use γ –irradiation as an eco-friendly method in silver nanoparticle preparation. The size and quality of prepared silver nanoparticles were proved by different techniques (EDX, TEM, UV, XRD). It could be suggested that AgNPs can diminish the challenges of using Gemcitabine in HCC treatment via enhancing the intrinsic and extrinsic apoptotic pathways as well as suppression of SMAD4 and miR-21 expression. Finally, this work highlighted that using AgNPs as a co-treatment with chemotherapeutics such as GEM accomplished of providing greater therapeutic effects than single-method treatment which is an advanced therapeutic strategy.

**Declarations**

**Ethical declaration**

The study followed the recommended guidelines for the use and care of laboratory animals of the National Institute of Health (NIH publication no. 85-23, revised 1996) and regulations of the Ethical Committee (REC) of the NCRRRT, Atomic Energy Authority, Cairo, Egypt (Approval No: 10 A/22). REC has approved this research protocol, following the 3Rs principles for animal investigation (Replace, Reduce, and Refine), and is operated and organized as per the CIOMS and ICLAS International Guiding Principles for Biomedical Research Involving Animals 2012.

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Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Consent to participate

All authors agree to participate in this research study.

Consent to Publish

We Soheir A. Osman, Shadia A. Fathy, Mohamed R. Mohamed, Amany I. Raafat, Mahmoud M. Refaat, Asmaa A. Hassan give our consent for information about all to be published in the Journal of Cancer Research and Clinical Oncology

[Corresponding author] Asmaa A.Hassan

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81. Statements of Declarations

82. Ethical declaration

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Figures

Figure 1

UV absorption spectrum of the obtained AgoNPs.

Figure 2

Diffraction pattern and TEM image of the obtained AgoNPs.
a) X-ray diffraction patterns of the obtained b) TEM image of the prepared Ago NPs

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Figure 3

EDX spectrum of the obtained Ago NPs.

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Figure 4

MTT cytotoxicity assay (% of cell viability, % of cell toxicity and IC50) of the chemotherapeutic drugs on Wi38 cell (the value displayed as means ± SE (n=3) of two independent experiments.)
Figure 5

MTT cytotoxicity assay (% of cell viability) and IC50 of different chemotherapeutic drugs on HepG2 cells (the value displayed as means ± SE (n=3) of two independent experiments.)
Figure 6

Liver function enzyme activities of serum (AST, ALT, and ALP (U/L)) in different male rat groups. Each value represents the mean ± SE (n=7). Columns over headed with different letters are considered significantly different (p<0.05). a: different from control, b: different from Ag, c: different from G; and d: different from T.
Figure 7

cells Apoptotic analysis of tested cells. The overall percentage of viable cells and dead cells comprises the total apoptotic cells (early and late) as well as the necrotic: a- normal control (C), b- HCC developed group(T), c- Animals developed HCC & treated with AgNPs (TAg) , d- HCC developed rats & injected with Gem, e- HCC developed rats & treated with AgNPs and Gem(TAgG) Data are mean ± SE, at p < 0.05.
Figure 8

Forward scatter (FSC) vs. side scatter (SSC) plots after doublet exclusion. (a) Control (C); (b) HCC (T); (c) HCC-AgNPs treated group (TAg); (d) HCC-Gem treated group (TG); (e) HCC-AgNPs- Gem treated group (TAgG).

Figure 9

The relative gene expression of CYT-P450 m-RNA in different examined groups. Each value represents the mean ± SE (n=7). Columns over headed with different letters are considered significantly different (p<0.05). a: different from control, b: different from Ag, c: Different from G; and d: Different from T.
Figure 10

The protein expression of different apoptotic markers (Bax, BCL2, and Caspase9) in all examined rats. Each value represents the mean ± SE (n=7). Columns over headed with different letters are considered significantly different (p<0.05). a: different from control, b: different from Ag, and c: Different from G; d: Different from T.

Figure 11
The protein expression of SMAD 4 in all examined rats. Each value represents mean ± SE (n=7). Columns over headed with different letters are considered significantly different (p<0.05). a: different from control, b: different from Ag, c: Different from G; and d: Different from T.

Figure 12

The relative gene expression of miR-21 in different examined groups. Each value represents the mean ± SE (n=7). Columns over headed with different letters are considered significantly different (p<0.05). a: different from control, b: different from Ag, c: Different from G; and d: Different from T.
Figure 13

Photomicrograph of liver tissue sections. (a): Normal Control liver section. (b): Ag group showed normal sized portal tract (blue arrow) surrounded by cords of average sized hepatocytes with no degeneration or necrosis (red arrow), (c): G group showed central veins (red arrows) surrounded by degenerated hepatocytes, some of them showed spotty necrosis (blue arrows), (d): AgG group showed mild dilated central vein (blue arrow) surrounded by cords of mild degenerated hepatocytes, some showing pyknotic (dark stained small) nuclei (red arrows) and apoptotic bodies (black arrow), (e): HCC group showed sheets of malignant hepatocytes exhibiting pleomorphism, hyperchromatia and abnormal mitotic figures (blue arrows) and focal areas of necrosis (red arrows), (f) TAg group showed malignant cells (red arrow) surrounded by 80% of tumor necrosis (blue arrow), (g): TG group showed malignant cells (black arrow) surrounded by 60% of tumor necrosis (red arrow), and (h): TAgG group showed few residual malignant cells (blue arrow) surrounded by 95% of tumor necrosis (red arrows).