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Research Article

Evaluation of the Potential Role of Silver Nanoparticles Loaded with Berberine in Improving Anti-Tumor Efficiency

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Abstract

Background: Cancer is a progressive disease, its incidence and death rates are rapidly increasing globally. Numerous adverse effects are associated with the available interventions. Hence, the current study was undertaken to explore the anticancer effect of silver nanoparticles conjugated with berberine (AgNPs-BER) against Ehrlich solid carcinoma (ESC) in mice.

Methods: Male Swiss albino mice were allocated randomly into ESC, ESC+cisplatin (CP; 5 mg/kg), ESC+AgNPs-BER (20 mg/kg), and ESC+cisplatin and AgNPs-BER groups.

Results: AgNPs-BER administration increased significantly the survival rate and decreased body weight and tumor size as compared to ESC group. Additionally, AgNPs-BER enhanced the development of oxidative stress in the tumor tissue as indicated by the increased lipid peroxidation (LPO) and nitric oxide (NO) accompanied by a decrease in the examined antioxidant proteins (glutathione (GSH) and its derived enzymes along with superoxide dismutase and catalase). AgNPs-BER was found also to trigger apoptotic cascade in the tumor cells through upregulating the mRNA expression of the pro-apoptotic proteins (*Bax* and *caspase-3*) and downregulating the mRNA expression of the anti-apoptotic protein (*Bcl-2*). Moreover, AgNPs-BER improved partially the histopathological alterations in the developed tumor tissue as compared to ESC group.

Conclusion: Collectively, AgNPs-BER could be applied as an antitumor agent due to its pro-oxidant, pro-apoptotic, and antiangiogenic effects.

Introduction

Cancer is a progressive disease and considered as one of the most important causes of death in the world. Various environmental, social, cultural, lifestyle, hormonal and genetic variables could be associated with the development of cancer.[1] Breast cancer is one of the most common malignancies worldwide with a high curable percentage in patients with early-stage, non-metastatic disease. Meanwhile, the current chemotherapeutic agents and anti-hormonal treatment aren't effective in advanced cases with distant organ metastases.[2] Ehrlich tumor-bearing mice clinically resemble human breast tumors and are frequently used to evaluate the novel anti-tumor agents.[3] Chemotherapy is the most efficient and commonly used treatment for different types of tumors including breast cancer.[4] Currently, there are more than 130 nonspecific target anti-tumor drugs available in the market, however, their application is associated with several adverse reactions such as nausea, fatigue, weakness, vomiting, hair loss, memory impairments, and in some severe cases may lead to death.[5]

Consequently, discovering new effective alternative anti-tumor drugs with fewer side effects is urgently required. Indeed, natural products represent a vigorous exporter for the development of anticancer agents.[6] Berberine is an alkaloid found in various plant species that have been used widely in Ayurvedic and Chinese medication.[7] In folk medicine, berberine is used as an antibacterial,[8] antidiarrheal, and antiprotozoal agents.[9] Additionally, numerous studies showed its ameliorative impact against neurological,[10] gastrointestinal,[11] cardiovascular[12] and metabolic[13] diseases.

A growing number of reports have recently confirmed that the novel herbal extract berberine can kill cancer cells.

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In this regard, berberine exerted anti-cancer effects against lung, cervical and liver cancers, in addition to leukemia, and other malignancies.[14-16] Despite its safety, berberine still has hydrophilic nature, low bioavailability, and bioabsorption (0.4%) which seriously limits its application and development as a pharmaceutical preparation.[17]

Nanotechnology has grown rapidly in recent years, affecting a variety of areas including medical diagnostics and therapeutics.[18] Due to its consistency, economic synthesis, and fundamental characteristics, silver nanoparticles (AgNPs) have received great attention in medicine.[19] Previous reports demonstrated that AgNPs have numerous pharmacological features like antioxidant, antimicrobial,[20] and anti-inflammatory.[21]

AgNPs have gained significant attention in cancer research because of their simple synthesis and surface adjustment, robust increases, and superior biocompatibility.[22-23] There is an evolving need to create eco-friendly types of nanoparticles (NPs) that do not use hazardous chemicals in formulation. In the last decade, enormous attempts have been made to merge green chemistry and biological methods for synthesizing AgNPs. This study aimed to use Ber as an eco-friendly material in preparing AgNPs, and at the same time, we can take the advantage of AgNPs as a carrier for Ber to overcome its poor bioavailability. We also examined the potential cytotoxic and antitumor effect of AgNPs conjugated with Ber using Ehrlich solid tumor-bearing mouse model.

Materials and Methods

Preparation of AgNPs-BER

For an effective synthesis of biogenic AgNPs, the updated approach of El-Khadragy *et al.*[24] was adapted. In brief, a total of 5 ml aqueous solution of Ber (0.1 mM/ml) was applied to 0.1 mM/ml $AgNO_3$ and was stirred at 45–50 °C. A Zetasizer (ZEN 3600) was used to analyze the size of AgNPs-BER.

Animals and experimental design

Forty female Swiss albino mice aged 1.5–2 months old and weighed 16–23 g were obtained from the National Research Center (NRC), Cairo, Egypt. Mice were fed a regular pellet diet and given free access to water and were housed in standard conditions of temperature and humidity for one week for acclimation. ESC cells derived from the NRC and greater than 99 percent of cells were viable by trypan blue dye exclusion.[25]

ESC cells were embedded subcutaneously in the left legs of the animals by injection of 0.2 ml of 2×10^6 tumor cells in normal saline. The mice were arbitrarily divided into four groups (n=10). The control group (ESC) consisted of saline-taking mice only during the entire study. The AgNPs-BER group consisted of animals getting an oral aqueous solution of AgNPs-BER (20 mg/kg/d) for 10 days beginning on the 5th day of ESC cells injection. The selected dose of AgNPs was based on the previous study of Rageh *et al.*[19] CP group, on the tenth day after ESC inoculation, mice were given a single I.P dose of 5 mg/kg cisplatin according to Almeer *et al.*[4] CP+AgNPs-BER group, animals received both CP and AgNPs-BER at the definite dosages and schedules. On the 16th day, ether was used to anesthetize 7 mice from each group. Mice were killed by cervical dislocation after blood collection through heart puncture. For biochemical tests, blood was centrifuged at 3500 rpm for 22 minutes and serum was stored at -20 °C. Tumor tissue was gently removed, weighted, and separated into parts. For histopathological analysis, one part was settled in 10% formalin, while the others were frozen at -80 °C for further research. The remaining 3 mice from each group were left for calculation of MST.

This study was reviewed and approved by the institutional animal care and use committee. Faculty of Science, Helwan University (number HU/2019/Z/AEO0319-01). It was also performed in agreement with the European Community Directive (86/609/EEC).

Calculation of total changes in body weight

All groups of animals were weighed on day 0 and day 16. The percentage weight gain was determined using the equation:

%Weight gain = [(mice weight on 16th day/mice weight on day 0)− 1] × 100.[25] **Eq. 1**

Calculation of the tumor volume (TV)

Using the Vernier caliper, TV was determined after the 5th day of the treatment by the following equation:

$TV\ (mm^3) = 4\pi\ (A/2)^2 \times (B/2)$ **Eq. 2**

Where A and B are the minor and major tumor axes.

Determination of the median survival time (MST)

MST was calculated by the equation:

MST= [first death + last death in the group]/2. **Eq. 3**

The increase in median life span (% IMLS) was determined by the equation:

% IMLS= (MST of treated mice − MST of CON) × 100]/ MST of CON.[4] **Eq. 4**

Oxidative stress markers

GSH, LPO, and NO levels in ESC homogenate were determined by kits purchased from G-Biosciences Geno Technology Inc., USA Company according to the manufacturer techniques.

Antioxidant markers

The levels of glutathione reductase (GR), glutathione peroxidase (GPx), SOD, and CAT in ESC tissues were estimated using the methods mentioned by Factor *et al.*,[26] Weydert and Cullen,[27] Sun *et al.*,[28] and Luck,[29] respectively.

Molecular assay for the expression level of caspase-3 (Casp-3), Bax, and Bcl-2

Total RNA was extracted from ESC tissue and reverse transcriptase was used to make cDNA. On an Applied Biosystems 7500, RT-PCR processes were conducted using

Table 1. Primer sequences and accession numbers of *Bcl2, Bax* and *Casp3* genes.

Gene	Forward primer Sequence (5'->3')	Reverse primer Sequence (5'->3')
GAPDH	AATGGGCAGCCGTTAGGAAA	GCGCCCAATACGACCAAATC
BCL2	CCTATCTGGGCCACAAGTGAA	ACAGCCTGCAGCTTTGTTTC
BAX	CATGGGCTGGACATTGGACT	AAAGTAGGAGAGGAGGCCGT
CASP3	GCGGATGGGTGCTATTGTGA	ACACAGCCACAGGTATGAGC

Power SYBR® Green (Life Technologies, CA) and GAPDH was used as a housekeeping gene. Table 1 lists the primer sequences.

Determination of angiogenin (Ang) and vascular endothelial growth factor (VEGF) in ESC homogenates

The levels of Ang and VEGF in tumor homogenate were determined using ELISA kits from BioVendor (Gunma, Japan), according to the company's procedure.

Histopathological examination

Hematoxylin and eosin were used to stain parts of tumor tissue (4 µm thick) which were then viewed using Olympus BX 41 microscope (Japan). Microphotographs were analyzed for the following histomorphometric parameters: tumor necrosis area percentage at five random fields. Then, the average and standard deviation were calculated.

Statistical analysis

All results were stated as the mean ± standard deviation. Data were examined for multivariable correlations by ANOVA program. Duncan's test has been used as a post hoc test to compare significance between groups, while probability level of ($P<0.05$) was regarded as significant.

Results

AgNPs-BER characterization

AgNPs-BER was characterized with an average diameter of 26.4±3.1 nm and a mean zeta potential of -3.7 mV (Figure 1). The result of FT-IR analysis of synthesized AgNPs-BER is also depicted in Figure 1. A broad peak observed at 3307.87 cm^{-1} corresponds to O–H group. The absorption peak at 2128.01 cm^{-1} corresponds to C–H stretch alkynes. The band at 1635.65 cm^{-1} is due to C–O asymmetric stretch carbon compounds. The peak at 1391.47 cm^{-1} is attributed to the C–N stretching of the amines. C–X stretching in alkyl halides causes a band at 519.35 cm^{-1}. This result showed the existence of different functional groups that could be essential for both the reduction and stability of the AgNPs-BER.

Effect of AgNPs-BER on the body weight, survival rate, and tumor volume

In this study, the antitumor activity of Ber conjugated with AgNPs was examined in vivo regarding CP as a reference antitumor drug. Treatment of mice with CP or AgNPs-BER significantly ($P < 0.05$) decreased the tumor proliferation as indicated by the reduction in body and tumor weights and tumor size as compared to ESC group. A further decline in the tumor growth was observed in the combination therapy

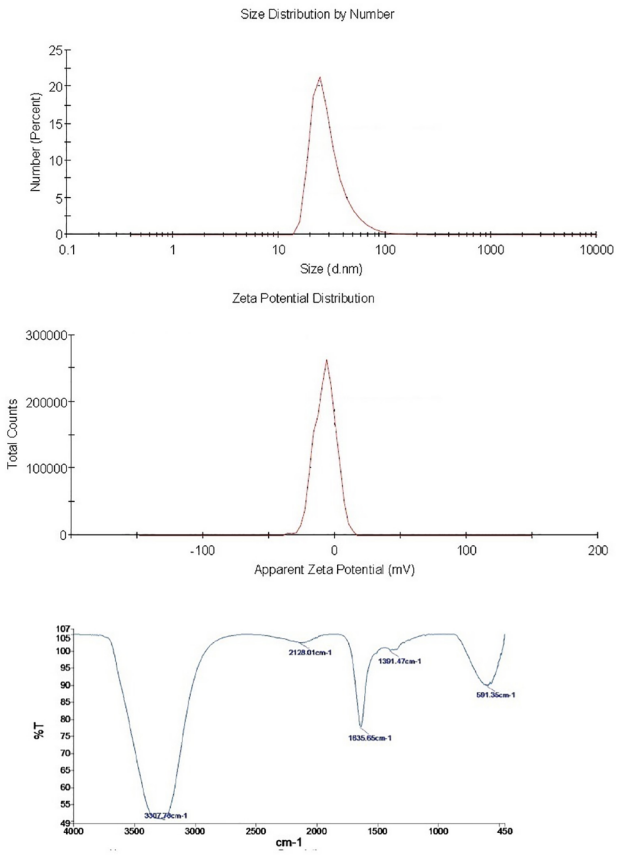

Figure 1. Characterization of berberine coated silver nanoparticles (AgNPs-BER). (Top panel) Hydrodynamic diameter of AgNPs-BER by Zeta sizer. (Middle panel) Surface charge of AgNPs-BER by Zeta potential. (Bottom panel) FT-IR spectra of AgNPs-BER.

group (CP and AgNPs-BER) compared with the control ESC as well single-treatment groups ($P < 0.05$; Figure 2). Moreover, AgNPs-BER alone or in combination with CP was found to prolong significantly MST from 15 days in ESC group to 26 days and 23 days respectively, while a non-significant change was recorded in mice treated with CP. Furthermore, treatment with AgNPs-BER or combination therapy significantly elevated ($P < 0.05$) the percentage of IMLS as compared with that of ESC group (Figure 2); reflecting the antitumor activity of AgNPs-BER.

Effect of AgNPs-BER on the oxidative status

As illustrated in Figure 3, CP and AgNPs-BER and their combination increased significantly ($P< 0.05$) MDA and NO levels in the tumor homogenate. Additionally, a significant decline in the antioxidant indicators (GSH, GPx, GR, SOD, and CAT) was recorded as compared to untreated mice. In addition, the combination therapy alerted MDA and NO levels and the activity of the

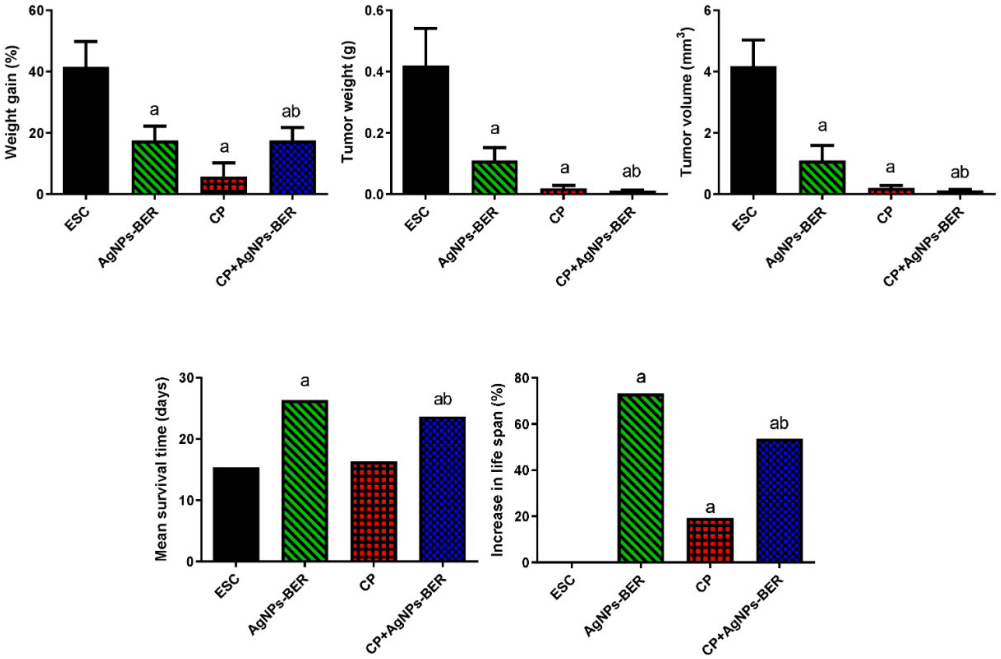

Figure 2. Effects of CP, AgNPs-BER, and CP+AgNPs-BER administration on body weight, survival rate and tumor volume in ESC-bearing mice. The obtained results are presented as means ± standard deviations (SD) (n = 7). [a]p < 0.05 compared to the ESC group (control group); [b]p < 0.05 compared to CP group.

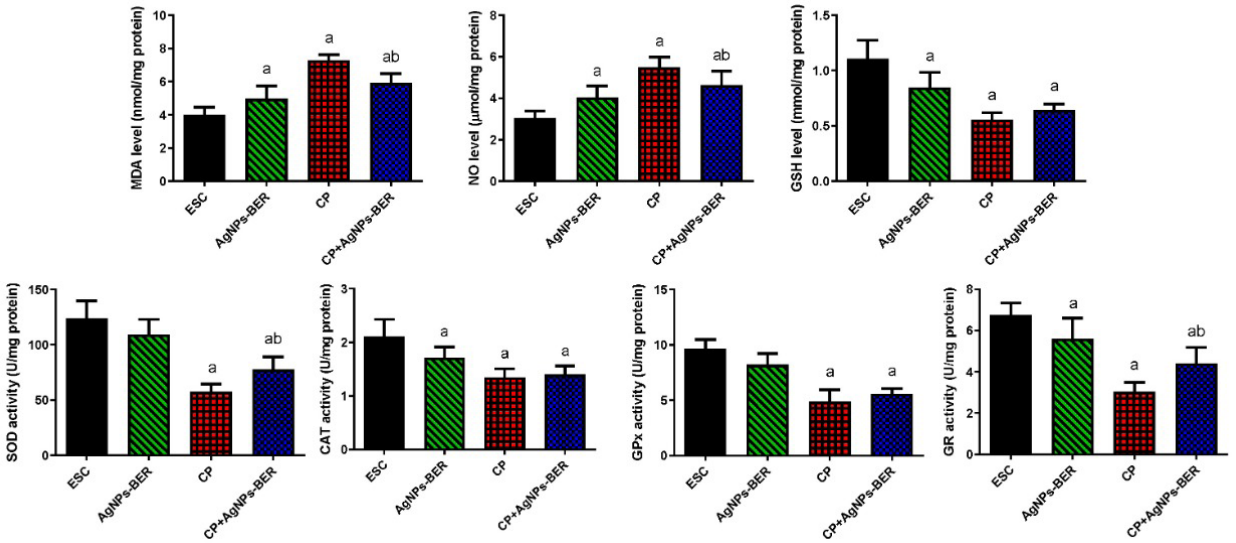

Figure 3. Effects of CP, AgNPs-BER, and CP+AgNPs-BER administration on oxidants (MDA and NO) and antioxidants (GSH, GPx, GR, SOD and CAT)] in ESC-bearing mice. The obtained results are presented as means ± standard deviations (SD) (n = 7). [a]p < 0.05 compared to the ESC group (control group); [b]p < 0.05 compared to CP group.

antioxidant indicators significantly (P < 0.05) compared to either treatment lonely.

Effect of AgNPs-BER on apoptotic proteins

The apoptosis-related genes (*Bax, Bcl-2, and Casp-3*) expression levels were investigated to evaluate the molecular pathway of antitumor activity of AgNPs-BER on ESC tissue. CP and AgNPs-BER and their combination upregulated significantly (P< 0.05) the expression of the pro-apoptotic proteins including Casp-3 and Bax, while downregulated significantly the anti-apoptotic protein, Bcl-2 as compared to their relative expressions in ESC group. Moreover, The combination therapy significantly (p<0.05) increased Bax and Casp-3 expression compared with CP group also (Figure 4).

Effect of AgNPs-BER on Ang and VEGF

In mice treated with AgNPs-BER, Ang and VEGF levels were significantly reduced (72.5 % and 63.5 %, respectively) in tumor tissue as compared to the CON

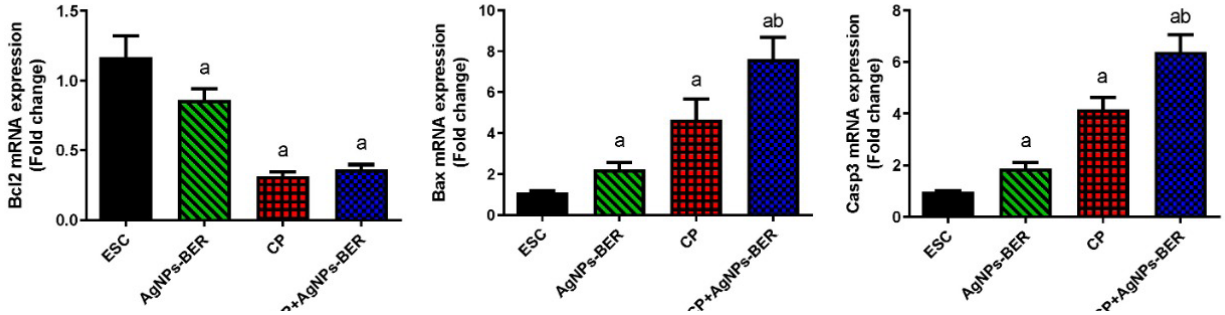

Figure 4. Effects of CP, AgNPs-BER, and CP+AgNPs-BER administration on mRNA expression of apoptotic proteins (Casp-3, Bax and Bcl-2) in ESC-bearing mice. The obtained results are presented as means ± standard deviations (SD) (n = 7). [a]$p < 0.05$ compared to the ESC group (control group); [b]$p < 0.05$ compared to CP group.

Table 2. Levels of angiogenin and VEGF in the studied groups.

Item	ESC group	CP group	AgNPs-Ber group	CP+AgNPs-BER group
Angiogenin (pg/mg protein)	587±54.3	278.8±30.4[a]	161.4±13.8[ab]	116.5±8.5[ab]
VEGF (ng/mg protein)	643.2±43.2	375±28.2[a]	234.8±25.4[ab]	197.4±14.9[ab]

The obtained results are presented as means ± standard deviations (SD) (n = 7). [a]$p < 0.05$ compared to the ESC group (Control group); [b]$p < 0.05$ compared to CP group.

(P < 0.05). In comparison to the control ESC group, CP treatment significantly reduced Ang and VEGF levels by 52.5 and 41.7 %, respectively (P < 0.05). When compared to the CP group, the combination group had significantly lower levels of Ang and VEGF (P < 0.05; Table 2).

Effect of AgNPs-BER on histological alterations

Injection of ESC cells into normal mice caused the development of the tumors at the point of injection. As seen in Figure 5a, Tissue samples from untreated ESC mice revealed condensed and accumulated malignant cells, along with groups of big, circular, and polygonal cells with pleomorphic shapes and binucleation. CP-treated mice also had necrotic cells, and residual cancer cells surrounded the muscle tissue (Figure 5b). Further damage of the ESC tissue was noted following the treatment with AgNPs-BER or combination therapy (Figures 5c and 5d). The tumor was discontinuous and seemed to be growing sluggishly and scattered in these two groups. These results were also confirmed with histomorphometric analysis; tumor

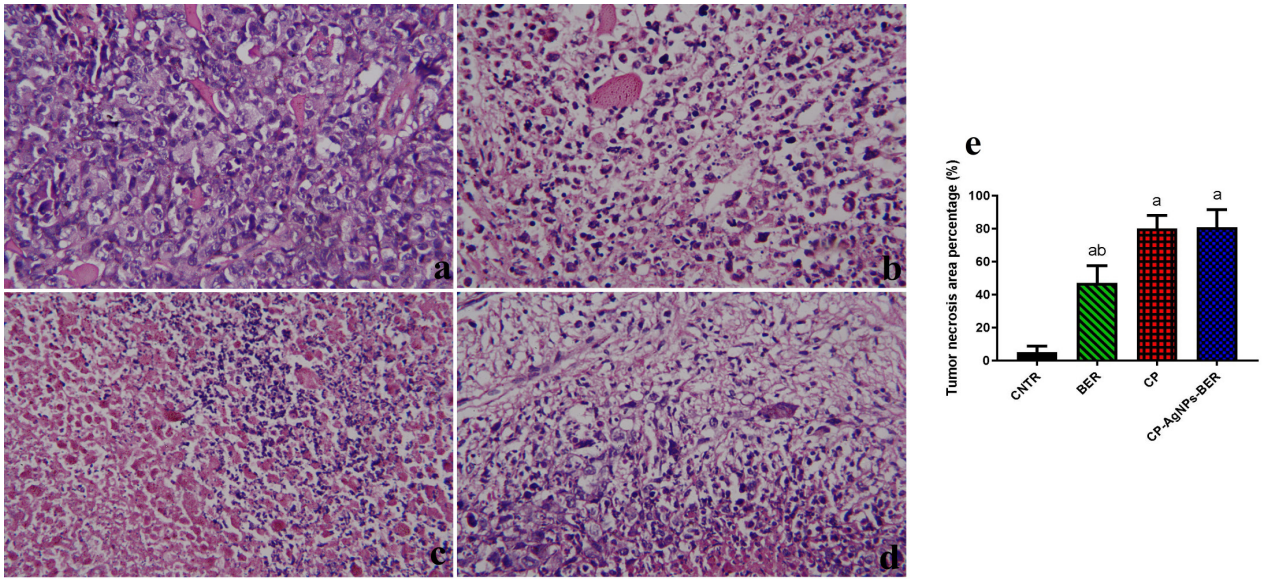

Figure 5. Histopathology of ESC tumors in mice treated with saline (a), CP (b), AgNPs-BER (c), and CP+AgNPs-BER (d). Microphotographs of Ehrlich solid tumor of saline-treated group revealed large, round, and polygonal cells, with pleomorphic shapes, hyperchromatic nuclei, and binucleation. Treated Ehrlich solid tumor with CP, AgNPs-BER or CP+AgNPs-BER showed a high regression of tumor development, spread within the muscle tissue, wide zones of apoptotic cells, and many zones of tumor cell remnants. (e) Histomorphometric parameters of ESC tumors in different treated mice: Tumor necrosis area percentage. The obtained results are presented as means ± standard deviations (SD) (n = 5). [a]$p < 0.05$ compared to the ESC group (control group); [b]$p < 0.05$ compared to CP group.

necrosis area percentage (Figure 5e) was significantly decreased in CP, AgNPs-BER or CP+AgNPs-BER-treated group compared to control ESC group ($P < 0.05$).

Discussion

In order to explore a novel anticancer drug with low side effects and high efficiency based on nanotechnology, the present study was undertaken to investigate the potential antitumor properties of AgNPs-BER against ESC in mice. The results of this study revealed a marked reduction in body and tumor weights and tumor size following the administration of AgNPs-BER. Furthermore, mice given a mixture of AgNPs-BER and CP had a significant decrease in tumor volume than mice given either drug alone. Additionally, the tumor growth was decreased, discontinuous and fragmented after administration of AgNPs-BER reflecting its ability to suppress the growth of ESC. These results were in line with that of El Bialy *et al.*[30] who stated that AgNPs decreased the body weight and size of Ehrlich solid tumor. In the same context, AgNPs administration in different doses was found to fragment tumors and consequently decrease their size.[19] Many studies support our findings that berberine exerted a cytotoxic effect against ESC in mice as evidenced by the decreased tumor growth and the increased MST and percentage increase in lifespan.[4,31] The authors referred this action to the induction of apoptosis following the treatment with berberine. It is well known that the malignant cells preferentially absorb higher amounts of NPs compared with the normal tissues due to their increased permeability and retention effect. Additionally, the ESC's weak lymphatic drainage allows NPs to infiltrate and remain in cancer cells. This can boost the targeted delivery of AgNPs to drugs.[32]

A high ROS generation promotes serious cell damage that leads to cell death. Oxidative stress induction is a promising technique because cancer cells are highly sensitive to ROS.[4] Our findings recorded a significant rise in MDA and NO along with a decrease in the endogenous antioxidants including GSH, CAT, SOD, GR, and GPx in the tumor tissues following the treatment with AgNPs-BER alone or in combination with CP.

It is clear from these results that the antitumor activity of AgNPs-BER may attribute to its pro-oxidant activity. Whereas, AgNPs exhibited different degrees of in vitro antitumor activity with different tumor cell lines.[33] AgNPs can penetrate cells, develop ROS and suppress antioxidant molecules, due to their small size and large surface area. While Ber also stimulates oxidative stress in tumor tissues by augmenting ROS production rate.[34] Ber thus synergizes with AgNPs and potentiates its oxidative stress action.

Crosstalk between oxidative stress, apoptosis, and the proliferation of ESC cells has been confirmed.[35] Carcinogenesis is associated with an imbalance between cellular proliferation and apoptosis. Enhancing apoptotic events has been attributed to suppressing the growth of potentially cancerous tumor cells.[4] Controlling tumor progression through potentiating programmed cell death is thought to be the key mechanism involved in malignant cell death following anticancer interventions. Casp-3 and Bax are essential in the execution process of cell apoptosis and have been linked to breast cancer apoptosis rates.[36]

The obtained findings showed that AgNPs-BER enhanced apoptotic cascade in the tumor cells as seen by the increased expression of pro-apoptotic proteins, Bax and Casp-3, and the decreased expression of the anti-apoptotic protein, Bcl-2. As a result, mitochondrial cytochrome c is released into the cytoplasm, activating caspase-9 and -3 which is crucial in performing apoptosis.[4] Recent studies reported that Ber can enhance tumor cell death through activating pro-apoptotic proteins and cell cycle arrest.[37] In a related way, Gurunathan *et al.*[38] pointed out that apoptosis is the main process by which AgNPs destroy breast cancer cells. Where AgNPs activate Casp-3 and endonuclease, this enhances the fragmentation of DNA and distinguishes apoptosis.[39] AgNPs were found also to enhance apoptotic cascade and block the pathway that triggers Dalton's lymphoma cells growth and viability.[38]

Furthermore, relative to animals treated with CP alone, the combined therapy (CP and AgNPs-BER) stimulated higher Bax and Casp-3 expression rates, which indicates more cancerous cell apoptosis. Angiogenin and VEGF are by far the most well-known angiogenesis initiators. Our results revealed that the levels of Ang and VEGF in malignant tissues were markedly decreased in AgNPs-BER group, which indicated that the anti-tumor effect of AgNPs-BER is mediated in part by reducing the levels of Ang and VEGF. Furthermore, AgNPs-BER amplified the inhibiting effect of CP on both Ang and VEGF. These results corroborated Hamsa and Kuttan's[40] findings that berberine therapy suppressed tumor-directed capillary creation as well as many pro-angiogenic agents such as Ang and VEGF.

Conclusion

In conclusion, by inducing oxidative stress and apoptotic cascades, and suppressing proangiogenic factors, AgNPs-BER may inhibit the growth of ESC in mice. The therapeutic effectiveness of cisplatin can also be enhanced by berberine-loaded AgNPs. This effect will efficiently reduce cisplatin doses and consequently its side effects. Further studies of AgNPs-BER anti-cancer mechanisms are important in order to produce an anticancer drug that is effective, safe, and economical.

Ethical Issues

This study was reviewed and approved by the institutional animal care and use committee. Faculty of Science, Helwan University (number HU/2019/Z/AEO0319-01). It was also performed in agreement with the European Community Directive (86/609/EEC).

Author Contributions

MSO and AEA conceived and designed research, and analyzed data. AEA conducted experiments and

contributed new reagents or analytical tools. STO, AHA, MAF, OME, and RBK wrote the manuscript. All authors read and approved the manuscript.

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Conflict of Interest

The authors report no conflicts of interest.

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