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Biophysical study to enhancement of apoptosis in colon cancer cell line using silver nanoparticles driven by extremely low frequency magnetic field and electroporation

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ABSTRACT

Extremely low frequency magnetic field (EMF) with an intensity of 0.01 mT is used to intensify the effect of the Silver nanoparticles (SNPs) as an anticancer agent. EMF stimulates the movement and vibration of SNPs that may enhance the apoptotic process in colon cancer (CaCo2 cell lines). Electroporation (EP) is used to improve targeting of cancer cells, where pulsed electric field (200V) creates hydrophilic gaps in cell membrane leading to elevated SNPs transport into colon cancer cells. CaCo2 cell lines are divided into 6 groups according to treatment type. Results show a clear accumulation during the G2/M phase of arrested cells accompanied by an extremely significant apoptotic percentage 7.59 % for cell lines treated with silver nanoparticles, electroporated and then exposed to electromagnetic field (0.01mT) for 1 hr (EP+ EMF+SNPs+Cells). A significant apoptotic percentage 4.12 % is also reported in the group: EP+SNPs+Cells. The lethal effect of the combination of EP, EMF and SNPs on CaCo2 cell lines is further reinforced by comparing the regulation of apoptotic genes (P53, Caspase-3, BCL-2) with its corresponding values in the control cells. The combination of EP, EMF and SNPs is probably a promising method for colon cancer treatment.

Key words: Colon cancer; silver nanoparticles; low frequency magnetic field; electroporation; CaCo2 cell lines.

INTRODUCTION

Cancer is one of the major diseases that affects human kind worldwide. It is known to be a complex, multifunctional disease due to its ability to spread in normal cells due to different genetic or environmental factors[1,2,3]. Colon cancer is known to be an aggressive kind of cancer as it occurs when the body lacks the ability to absorb water as well as salts from solid waste, hence leading to non-controllable overgrowth of cells in the large intestine. Known symptoms of colon cancer include constipation, diarrhea, Irritable Bowel Syndrome (IBS), and rectal diseases. Common, cancer therapies include chemotherapy, surgery, immunotherapy, hormone therapy, or radiation therapy, however, these methods lack directed delivery to cancer cells[4].

On the other hand, Silver nanoparticles have shown its ability to overcome the effects that accompany cancer therapies as it leads to direct drug therapy with few side effects[5]. In addition, silver has many properties that have been used for treatment of various diseases, for instance, it serves as an antibacterial or antimicrobial agent because of the well-developed surface of silver nanoparticles which provides the maximum contact with the environment[6]. Studies in the past years have shown that silver nanoparticles can be successfully used as an anticancer agent due to its anti-proliferation and apoptosis induction properties[7].

The present work investigates the incorporation of additional factor that may help in the apoptotic induction process in cancer treatment (electroporation, EP) and is reported to improve the efficacy of silver nanoparticles and it is use in specific cell targeting[8]. Using EP, cells are exposed to pulsed electric field resulting in abnormal increase in the permeability of the outer membrane. Hence EP is used in many applications due its ability to

cause temporary hydrophilic gaps in the cell, thus reducing transmembrane resistance and allowing the passage of drugs into the viable cells[9,10]. Another influencer in this study is the Low Frequency Magnetic Field (ELF-MF) or (EMF) as it has the ability to cause changes in the metabolism of cells when treated with precise frequencies and amplitudes. EMF is known for its ability to overcome cell proliferation caused by cancer cells as well as enhancing the apoptosis process[11,12]. Other existing works exposed cancer cells to nanoparticles with EMF, here the formed membrane pores by EP target specific cells that decrease the lethal side effect to normal cells.

Therefore, the aim of this study is to evaluate the use of electromagnetic field in conjunction with electroporation and silver nanoparticles in the treatment of colon cancer cells and to determine the way this method affects internal structures, genes and rate of apoptosis.

MATERIALS AND METHODS

Materials

Silver nitrate and tri-sodium citrate were purchased from Sigma Aldrich, USA. Cell line CaCo2 (Colon Cancer Cell line) was provided by the Tissue Culture Department, Vaccines & Sera (VACSERA), Egypt and Fetal bovine serum (FBS) was purchased from Invitrogen Corp., Carlsbad, CA. Phosphate-buffer saline (PBS) was purchased from ADWIA–Egypt. Penicillin-streptomycin, Trypsin, EDTA, and Hank's buffer were purchased from Gibco, USA. There was no further purification of the reagents.

Methods

1. Silver nanoparticles preparation

Silver nanoparticles (SNPs) were prepared by reduction procedure in low concentration, to minimize the toxic effect of SNPs as much as possible. Therefore, 0.0024 g of sodium nitrate/ 100 ml water was mixed drop wise with 0.029 g/ 50 ml of Tri-sodium citrate at 100 °C using a magnetic stirrer. The color change was observed as yellow color that would start to appear once silver nanoparticles were formed.

2. Identification and characterization of SNPs

Spectrophotometer (SHIMADZU QP2010, Japan) was used to test the absorbance of silver nanoparticles. It revealed an absorption peak at 450 nm, hence the concentration of the silver nanoparticles used was 7.45×10^{-9} m mol / L. In addition, Characterization of silver nanoparticle shape was carried out using Transmission Electron Microscope (TEM) and later, samples were stored in sterilized cuvette for further used.

3. Cell culture

Colon cancer cell lines (CaCo2) were provided by tissue from VACSERA, Egypt. CaCo2 cells were maintained as monolayer culture in RPMI-1640 medium supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin at 37 °C in a (5% CO_2) humid atmosphere. When the cells reached at least 80% confluence, they were washed twice with Phosphate-Buffer Saline (PBS) and were detached with 0.25% trypsin/EDTA in Hank's buffer. An equal volume of medium with FBS for trypsin inactivation was added and the tumor cells were collected and counted.

4. Electroporation (EP) and Electromagnetic field (EMF) treatments

The suspended CaCo2 cells (2×10^4 /2.5 ml) were seeded in cell culture petri-dishes (8 cm in diameter) and incubated at 37 °C (5% CO2). Cells were divided into 6 test tubes

according to the treatment type: Control CaCo2 cells, EP+Cells, EMF+Cells, EP+EMF+Cells, EP+SNPs+Cells and EP+EMF+SNPs+Cells, (Table 1). Using EP, cells were exposed to 10 pulses of 200V each at equal intervals of time by the electroporator (BIORAD, USA). The electromagnetic field intensity used was 0.01 Tesla. An amount of 0.5 µg/ml SNPs was mixed thoroughly with cells treated with SNPs then tested for cell cycle, apoptosis and molecular analysis including P53, BCL2, Casp-3, and Survuvin investigations.

Table 1. Details of the 6 different investigated groups.

Test Tubes	mV	mT
Control CaCo2	-	-
EP+Cells	200V	-
EMF+Cells		0.01
EP+ EMF+Cells	200V	0.01
EP+SNPs+Cells	200V	-
EP+ EMF + SNPs+ Cells	200V	0.01

5. Cell cycle analysis by flow cytometry

Cell cycle phase distribution was evaluated using a method described by Poolman and Brooks [9] with minor modifications. Cells were seeded in flask (1 × 106 cells/ flask), and treated with the IC50 of free sinapic acid and niosome encapsulating sinapic acid and incubated for 24h. The cells were then harvested, centrifuged, and fixed in ice-cold 70% ethanol. The fixed cells were again centrifuged and resuspended in 100 µL of PBS. The cells were incubated with RNase A and stained with a propidium iodide solution. The labeled cells were analyzed using a FACSCan flow cytometer (Becton Dickinson).

6. Apoptosis analysis

Induction of apoptosis in control, free sinapic acid and niosome encapsulating sinapic acid was assayed using flow cytometry according to the manufacturer's instructions flow cytometer laboratory protocol.

7. Statistical analysis

Data are analysed using the Duncan's multiple range test by SPSS software (version 17; SPSS, Chicago, IL). The differences are considered significant at $P < 0.001$

RESULTS

1. Identification and characterization of SNPs

UV-Visible absorption spectrum

Silver nanoparticles previously reported exceptional optical, electrical and thermal properties, where spherical and/or circular silver nano-particles with diameter smaller than 50 nm in size showed an absorption peak at 470-490 nm. The increase in the size of nanoparticles and their aggregation would lead to sensible broadening of the absorption peak and its shift towards higher wavelengths[13]. In the present work, the prepared SNPs reveal a peak at a wavelength of 455 nm (Fig. 1), reflecting that their size is probably smaller than 50 nm.

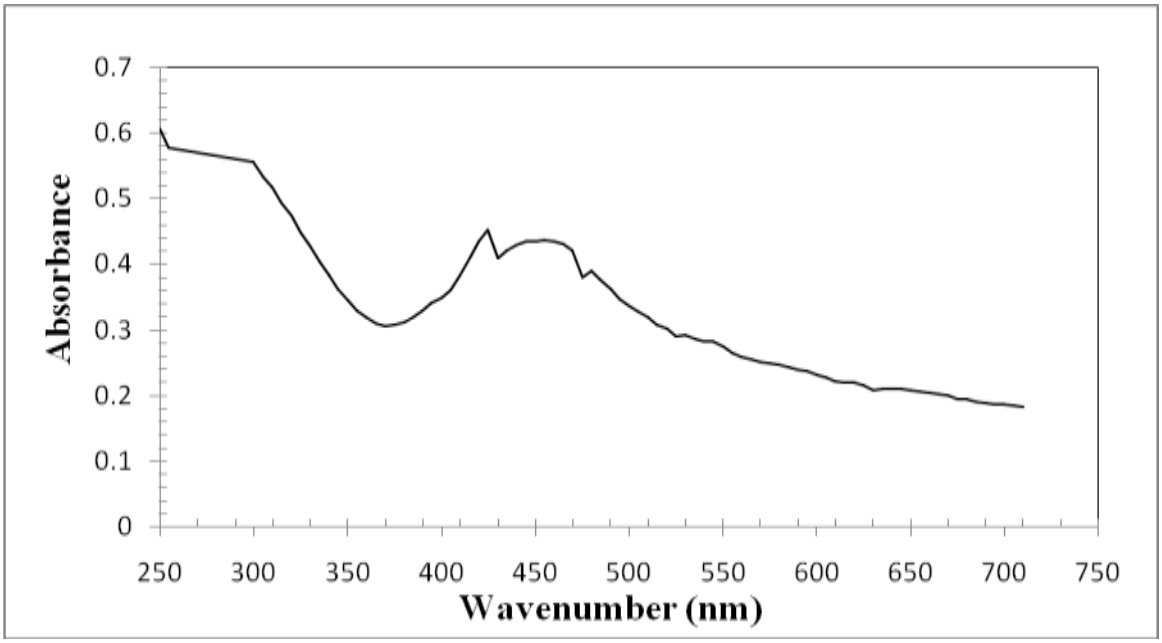

Fig. 1. UV-Visible absorption spectrum of SNPs.

Transmission electron microscopy

The examination of the ultra-structure using TEM shows that the prepared SNPs are homogenous, non-aggregated, spherical shape particles of comparable size (Fig. 2).

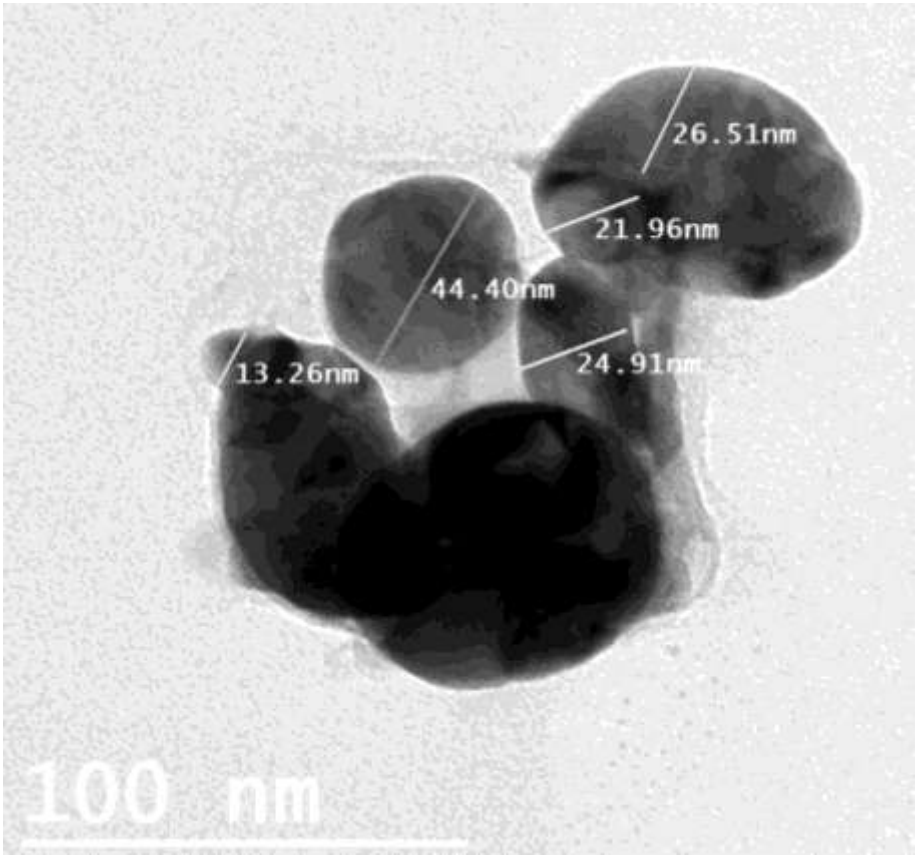

Fig. 2. TEM image of SNPs.

2 The treatments protocols on CaCo2 cell line

Human colon cancer cell line (CaCo2) treated using electroporation followed by one hour exposure to electromagnetic field (0.01mT) shows no effect on cell viability. The viability percentage is determined using MTT assay and shows a value ranging between 95-100%. Regarding the apoptotic profile induced in EP+SNPs+Cells and EP+EMF+SNPs+Cells, it is noticed that there are a clear accumulation of the arrested cells during the G2/M phase (Fig. 3a). The percentage of cell arrest is dependent on the presence of SNPs, where there is a significant ($P<0.05$ and $P<0.001$) arrest percentages for EP+SNPs+Cells and EP+EMF+SNPs +Cells groups, respectively (Fig. 3b). In the meantime, the cell arrest is accompanied by cell profile characterized by significant ($P<0.005$ and $P<0.0001$) apoptotic percentages for EP+SNPs+Cells and EP+EMF+SNPs+Cells groups, respectively. The arrest and apoptotic profiles of the other groups show insignificant changes ($P>0.05$) compared to control.

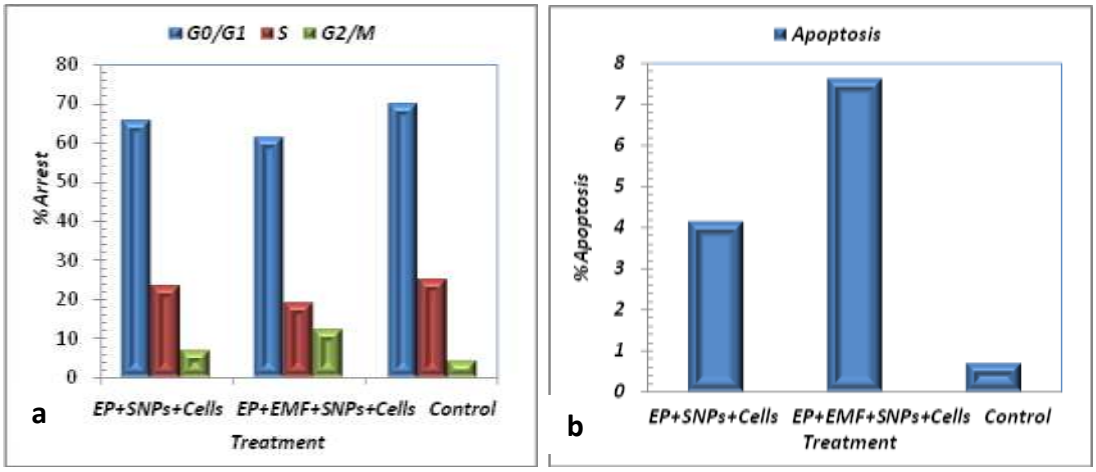

Fig. 3. (a) The variation of % arrest for cell cycles G0/G1, S and G2/M and (b) % apoptosis for EP+SNPs+Cells, EP+ EMF+ SNPs+Cells and control samples.

Regarding the apoptotic gene profiles induced in CaCo2 cells for groups EP+SNPs+Cells and EP+EMF+SNPs+Cells, it is noticed that there is a significantly ($P<0.05$) elevated up regulation of apoptosis P53 gene compared with control cells. A significant difference ($P<0.05$) exists in the up regulation rate for group EP+EMF+SNPs+Cells compared to group EP+SNPs+Cells. In the meantime, there is a significant ($P<0.05$ and $P<0.005$) increase in up regulation of Caspase-3 gene compared to control for EP+SNPs+Cells and EP+EMF+SNPs+Cells groups, respectively (Fig. 4). For BCL-2, the EP+SNPs+Cells group is insignificantly ($P>0.05$) down regulated compared to control, while the EP+EMF+SNPs+Cells group is significantly ($P<0.05$) down regulated compared to control. For survivin, both groups are significantly ($P<0.05$) down regulated compared to control. The down regulation in both groups are statistically similar.

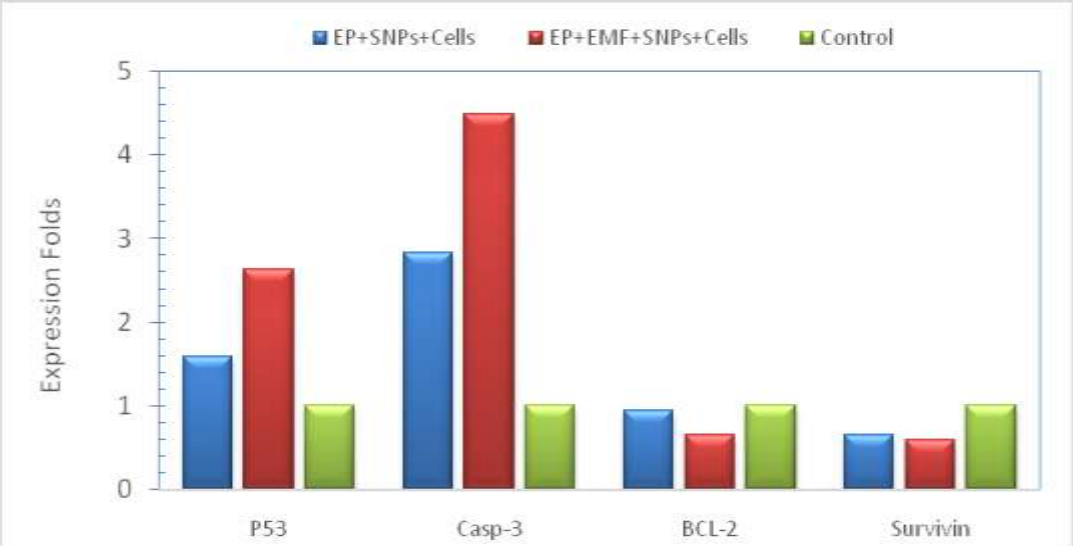

Fig.4. Apoptotic gene profile induced in EP+SNPs+Cells, EP+EMF+SNPs+Cells, and Control cells for Apoptotic genes (P53 and Casp-3) and Anti-Apoptotic genes (BCL-2 and Survivin).

DISCUSSION

The cytotoxic effect of SNPs has always attracted the attention towards possible application in cancer treatment[14,15]. Here we emphasize the lethal effect of SNPs on CaCo2 tumor cells. SNPs alone produce reactive oxygen species (ROS) and, therefore, are capable to induce apoptosis[6,16-19]. It has been reported also that SNPs activate signaling pathways that lead to the inhibition of cell proliferation by acting on membrane proteins[16,20,21]. It should be noted that silver nanoparticles can also enter cells through endocytosis where the high surface area of SNPs increases the interaction with fluid components compared with bulk particles producing mitochondrial dysfunction, destruction of proteins and nucleic acids, formation of reactive free radicals. All of these effects strongly enhance the inhibition of cell proliferation[6,16-19, 22].

This work also examines a novel approach involving the introduction of electroporation (EP) in order to facilitate the entrance of SNPs into tumor cells, in addition to the initiation of localized tumor cell damage[23]. Moreover, in order to maximize the potential effects of SNPs, EMF is applied in conjunction with EP. The exposure of cells to EMF is expected to enhance the formation of membrane pores as a result of interaction between the externally applied EMF and the magnetic properties of cell membranes[24].

ROS are generally considered one important effect of the application of various anticancer agents on cell cycle phases[25, 26]. It is reported that SNPs stimulate an elevation in the intracellular ROS[6,16-19]. Therefore SNPs induce apoptosis or cell cycle arrest by increased production of ROS. In the present work, EP+SNPs+Cells and EP+EMF+SNPs+Cells induce increased accumulation of cells in the G2/M phase in addition to increased cell apoptosis (Fig. 3) probably due to the increase in DNA damage caused by the increased production of ROS which, in turn, cause increased inhibition of proliferation in the CaCo2 cell lines. CaCo2 cells were most sensitive to EP+EMF+SNPs+Cells protocol.

Our findings demonstrate that the examined SNPs treatments may serve as protective agents against colon cancer, where it is observed that the apoptosis ratio increased to 4.12 % and 7.59 % in EP+SNPs+Cells and EP+EMF+SNPs+Cells treatments, respectively, compared to control cells 0.68%.

Biophysical study to enhancement of apoptosis in colon cancer cell line using silver nanoparticles driven by extremely low frequency magnetic field and electroporation

Apoptosis controlled cell death by a series of morphological events and is carried out by an active cellular process. One of the principal signaling transduction pathways involved in the process of apoptosis is the mitochondrial pathway[27]. In the present study it is initiated by the up regulation of p53 by 1.59 and 2.63 folds , followed by suppression of Bcl-2 by 0.95 and 0.65 folds in EP+SNPs+Cells and EP+EMF+SNPs+Cells treatments, respectively, compared to control cells. These results suggest that SNPs stimulates the reduction in mitochondrial transmembrane potential and the release of mitochondrial cytochrome c, finally activating the mitochondrial-mediated apoptosis pathway[15,28]. The activation of caspase-3 by 2.83 and 4.48 folds in EP+SNPs+Cells and EP+EMF+SNPs+Cells treatments, respectively is also reported. Caspases, a family of cysteine proteases, play essential role in apoptosis, necrosis and inflammation. The activation of caspase-3 induces apoptosis through the mitochondrial pathway[29]. In the present study, it is shown that SNPs enhance caspase-3 activity, indicating that the mitochondrial death pathway is involved in the process of apoptosis induced by SNPs' treatments.

Survivin is suppressed by 0.65 and 0.60 folds in EP+SNPs+Cells and EP+EMF+SNPs+Cells treatments, respectively. Survivin serves, in participation with other proteins, in the regulation of apoptosis, cell motility, and cell division[30]. The examined SNPs' treatments are shown to be able to suppress survivin function and induce cancer cell death. These treatment strategies could result in potential enhancement of the effectiveness of many drugs.

Treatment including EMF (EP+EMF+SNPs+Cells) with an intensity of 0.01 T is shown to produce increased damage to tumor cells. This is probably due to the ability of EMF in stimulating the movement and vibration of the SNPs that may enhance the apoptotic process. Despite electroporation-based treatment (EP+SNPs+Cells) produces enhanced cell damage due to the generation of temporary pores facilitating the entrance of SNPs across cell membrane, yet a combined treatment including both EP and EMF (EP+EMF+SNPs+Cells) clearly induces larger cell damage in CaCo2 cells due to the combined effect of EP and EMF.

There is no significant effect on tumor cells upon treatment by EP only under pulsed voltages of 200V and 260V (data not shown). A combination of SNPs and EP show a moderate enhancement in cell damage parameters compared to control.

Conclusion

In conclusion, we offer an in vitro study that examines the possibility to enhance specific CaCo2 cell damage through the application of SNPs combined to Ep+EMF. A highly evident intracellular damage is produced since EP induce membrane pores through the exposed cells. Therefore, this study strongly recommends a future in vivo application of such protocol for the treatment of CaCo2 cells.

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دراسة فيزيائية حيوية لتعزيز موت الخلايا المبرمج في خلايا سرطان القولون باستخدام جسيمات فضية نانوية مدفوعة بمجال مغناطيسي منخفض التردد للغاية والتثقيب الالكترونى

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المستخلص

يستخدم المجال المغناطيسي منخفض التردد للغاية بكثافة 0.01 تسلا لتكثيف تأثير الجسيمات النانوية الفضية كعامل مضاد للسرطان. يحفز المجال المغناطيسي منخفض التردد حركة واهتزاز الجسيمات النانوية الفضية التي قد تعزز عملية موت الخلايا المبرمج في سرطان القولون. يستخدم الثقيب الكهربى لتحسين استهداف الخلايا السرطانية ، حيث يخلق الحقل الكهربائي النبضي (200 فولت) فجوات ماء في غشاء الخلية مما يؤدي إلى نقل جسيمات الفضة مرتفع إلى خلايا سرطان القولون. وتنقسم خلايا سرطان القاولون إلى 6 مجموعات وفقا لنوع العلاج. تظهر النتائج تراكمًا واضحًا خلال المرحلة التمهيدية الثانية مصحوبة بنسبة تدمير ذاتى كبيرة للغاية 7.59 ٪ للخلايا المعالجة بالجسيمات النانوية الفضية وبالكهرباء ومن ثم تتعرض للحقل الكهرومغناطيسي (0.01 مللى تسلا) لمدة 1 ساعة (التثقيب الكهربى و المجال المغناطيسى منخفض التردد و جسيمات الفضة النانوية والخلايا). وقد لوحظ أيضًا وجود نسبة كبيرة لموت الخلايا المبرمج في المجموعة: التثقيب الكهربى و جسيمات الفضة النانوية والخلايا. تم استكشاف التأثير الفتاك لمزيج من التثقيب الكهربى والمجال المغناطيسى منخفض التردد و جسيمات الفضة النانوية على خلايا سرطان القاولون بمقارنة قيم الجينات المبرمجة لعملية التدمير الذاتى مع قيمها المقابلة في خلايا المجموعة الضابطة. وقد توصلنا الى ان الجمع بين التثقيب الكهربى والمجال المغناطيسى منخفض التردد و جسيمات الفضة النانوية يعد الطريقة الأرجح والواعدة لعلاج سرطان القولون.