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In Vitro Assessment of the Antineoplastic Activity of Doxorubicin Combined With Gemcitabine in a Nanoparticle

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Abstract

Objectives: The combination of 2 chemotherapeutic agents has been recommended in order to reduce their adverse side effects and potentiate their efficacy. The aim of the present study was to assess the antineoplastic activity of doxorubicin (DOX) combined with gemcitabine (GEM) in nanoemulsion (NE) against various cancer cells and to examine their adverse effects on the healthy human foreskin (HFS) cells.

Materials and Methods: The physical characterizations of the drug-loaded NE formulations were determined by Zetasizer. The cytotoxicity of the drugs was evaluated by the thiazolyl blue tetrazolium bromide (MTT) assay while the mechanism of cell death was examined by light microscopy, nuclear staining with 4′,6-diamidino-2-phenylindole (DAPI) and ApopNexin FITC apoptosis detection kit.

Results: The Zetasizer results demonstrated that the nanoparticle of the combination of 5 μM of DOX and GEM in NE (5DOX/5GEM-NE) has a particle size of 155.38 ± 3.08 nm with a polydispersity index of 0.02 ± 0.28 and a negative zeta potential of -7.70 ± 1.30 mV. The 5DOX/5GEM-NE has decreased the percentages of HeLa cervical cancer cell viabilities to 27.00 ±5.62%, but it has not considerably changed the percentages of HFS cell viabilities (97.06 ± 6.09%) when compared to the single treatments of DOX and GEM. According to the mechanism of cell death studies, 5DOX/5GEM-NE has induced apoptosis in HeLa cells without affecting the HFS cells.

Conclusions: The present study proved that formulating DOX and GEM in NE has ameliorated the efficacy of DOX and GEM as anticancer drugs while reducing their adverse effects on the healthy cells.

Keywords: Antitumor activity, Chemotherapeutic agents, Cytotoxicity, Apoptosis

Introduction

The combination of 2 anticancer drugs has been proposed by many researchers in order to formulate an agent with multiple targets that has the ability to overcome the cancer cell resistance (1,2). However, mixing the 2 drugs with different solubilities and bioavailabilities would be one of the most obstacles faced in the combination therapy (3). Therefore, it has been suggested that the nanocarriers as transporters for mixed drugs be employed due to their ability to deliver their cargo simultaneously into the cell and protect them from the enzymatic degradation inside the cell (4).

Nanoemulsions are colloidal systems that consist of nanoparticles with diameter range between 20 to 200 nm, produced by mixing oil, water with the aid of surfactants and/or cosurfactants (5). They have the ability to solubilize various kinds of drugs with different hydrophilicity. Both doxorubicin (DOX) and gemcitabine (GEM) are chemotherapeutic agents that are used to treat different kinds of tumors. DOX, an anthracycline with multi-molecular target potential, is mainly cardiotoxic and thereby there have been attempts to encapsulate it in different kinds of nanoparticles such as dendrimers, micelles and polymeric nanoparticles (6-8). GEM, an antimetabolite, has limited clinical outcomes and therefore, it was encapsulated in many nanocarriers in order to eliminate its adverse side effects (9-11). Mixing 2 chemotherapeutic agents (GEM and DOX) that have a different mechanism of actions would result in a synergistic effect on the cancer cells and thereby improving the efficacy of drugs at lower concentrations while reducing their side effects. The objective of this study was to assess the antitumor activity of DOX in combination with GEM encapsulated in nanoemulsion (NE) against various cancer cells (HeLa cervical cancer cells, HCT116 colon cancer cells and A549 non-small cell lung cancer cells) and to evaluate its side effect on the healthy HFS cells.

Materials and Methods

Chemicals and Cell Lines

DOX Hydrochloride and GEM hydrochloride were obtained from US Pharmacopeial Convention (USP).

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All of the constituents of the NE formula were purchased from Sigma (London, UK). The MTT assay was purchased from Biomatik (Ontario, Canada). DAPI stain was obtained from Invitrogen Life Technologies (New York, US). ApopNexin™ FITC Apoptosis Detection Kit was purchased from Millipore (Massachusetts, US). The human cervical cancer cell line (HeLa), human non-small cell lung cancer cell line (A549), human colon cancer cell line (HCT116) and human foreskin (HFS) cell line were procured from American Type Tissue Culture Collection (Manassas, VA, USA).

Methods

Formation of the Nanoemulsions Formulas

The NE formulation (Blank-NE) was produced, as mentioned before (12), by homogenizing 0.043 g of Eumulgin HRE 40, 0.043 g of sodium oleate and 0.037 g of L-α-Phosphatidylcholine followed by adding 0.15 g of cholesterol slowly. Finally, the mixture, containing 0.6 g of 1-octanol, was diluted with 99.15 g of Tris-HCl buffer (pH 7.22), vortexed, and incubated in the water bath at 75°C for 3 hours.

The drugs-loaded NE, selected according to a previous study (12), contained 5 µM of DOX (5DOX-NE), 5 µM of GEM (5GEM-NE), and a mixture of 5 µM of DOX and 5 µM of GEM (5DOX/5GEM-NE). Similarly, the solution formulations were produced by dissolving the same amount of drugs in water instead of NE and were designated as 5DOX-D.W, 5GEM-D.W, and 5DOX/5GEM-D.W.

Physical Characterization of Nanoemulsions Formulas Using Zetasizer

The z-average diameter of particles (nm), polydispersity index (PDI) and zeta potential (mV) of the selected NE formulations were determined by Malvern Zetasizer analyzer instrument. First, the z-average diameter was measured by photon correlation spectrometer using laser light scattering. A 1 mL of each sample was loaded onto cuvette and inserted into the thermostatic chamber. The z-average of particle size was determined. Second, zeta potentials of the NE formulations droplets were measured by laser Doppler electrophoresis. All samples were injected into capillary cells for charge measurement. Zeta potential values provide information on the repulsive forces between particles. All data were analyzed by Zetasizer version 7.2.0. All NE formulations were dispersed in DMEM solution.

Cell Cultur

All human cell lines were grown in a 25 cm^2 cell cultured flask, preserved in Dulbecco's modified eagle medium (DMEM), which was provided with 10% (v/v) heat inactivated fetal bovine serum, 1 % (v/v) penicillin streptomycin and incubated in a 95% air and 5% humidified CO_2 at 37°C. The media were changed every 48 hours until cells reached confluence. The confluent cells were washed with 2 ml of PBS and dissociated with 2 mL of trypsin. Cells were passaged every 3 days.

Cytotoxicity Screening Using MTT Assay

The MTT cell proliferation assay was used to testify the toxicity of solution and NE formulations against HeLa, A549, HCT116 and HFS cells. A 100 µL of culture media containing 5000 cells, counted using a countess automated cell counter (Invitrogen, US), was seeded in each well of 96-well plate and incubated overnight in 95% air and 5% humidified CO_2 at 37°C. Wells that contained free culture media were considered as the negative control, whereas wells that included culture media with the cultured cells without administering drug served as the positive control. Cells treated with 100 µL of NE and solution formulations were incubated for 24 hours. Following incubation, 5 µL of MTT reagent was added to each well and left for 3-4 hours at 37°C. Then, the culture media and MTT were removed, followed by the addition of 100 µL of DMSO and left for 2 hours at 37°C. Finally, the absorbance of each well of the plate was read at 540 nm using a microplate reader (BioTek, US). The percentages of cell viability were determined by the following equation:

$$Cellviability(\%) = \frac{Absorbance\ of\ treated\ cells - Absorbance\ of\ negative\ comntrol}{Absorbance\ of\ positive\ comntrol} \times 100$$

Detection of Apoptosis

A) Light microscopy: To investigate whether the treated HeLa and HFS cells have undergone apoptosis, light microscopy was utilized as described elsewhere (13).

B) Nuclear staining using DAPI: The blue-fluorescent, DAPI, is a nucleic acid stain that preferentially stains A-T base pair in the DNA. In a 24-well plate, 500 µL of media containing 50 000 cells was cultured for 24 hours. Then, the adherent cells were treated with the selected NE formulations and incubated for 24 hours. After that, cells were equilibrated with 300µl of PBS, fixed with 200 µL of formaldehyde and stained with 300 nm of DAPI solution for 1-2 minutes. Finally, the solution was discarded and the stained cells were observed by an inverted fluorescence microscope (Leica DM16000 B, Germany). The percentages of fluorescence intensity in each fluorescent microscopic image were estimated by ImageJ version 1.48.

C) ApopNexin™ FITC apoptosis detection assay: ApopNexin™ FITC apoptosis detection kit contains annexin V conjugated with fluorescein isothiocyanate (FITC, green fluorescence) which stains the phosphatidylserine (PS) that was normally located in the inner surface of the cell membrane of viable cells. The translocation of PS to the outer membrane is a sign of cell apoptosis. The counterstain, propidium iodide (PI, red fluorescence) stains the necrotic cells, which is used to discriminate the apoptotic cells from necrotic cells.

Cells were grown in a 24-well culture plate at a density of 50 000/well for 24 hours and then treated with the

selected NE formulations for 24 hours. Then, cells were washed with 300 µL of PBS, dissociated with 200 µL of trypsin and spin down at 1000 rpm for 5 minutes. After that, the supernatants were removed and cells were re-suspended in ice-cold PBS and spun down. The last step was repeated twice. Cells were re-suspended in 200 µL of 1x binding buffer followed by the addition of 3 µL of FITC and 2 µL of PI. Cells were incubated at 25°C in the dark for 15 minutes. All cells were evaluated by BD FACSAria III flow cytometer (BD Biosciences, US). Data were analyzed using FACSDiva software Version 6.1.3. The positive FITC indicates the release of PS, which happens in the early stage of apoptosis and the positive PI indicates necrotic cells.

Statistical Analysis

Statistical analyses were conducted with one-way analysis of variance (ANOVA) test using MegaStat Excel (version 10.3, Butler University). The Statistical significant differences between samples were considered according to the following guidelines: (1) If $0.01 \leq P < 0.05$, the difference between samples is significant (*); (2) If $0.001 \leq P < 0.01$, the difference between samples is highly significant (**); and (3) If $P < 0.001$, the difference between samples is very highly significant (***).

Results

Zetasizer Measurements for NE Characterization

As displayed in Table 1, the z-average diameter of the nanodroplet of 5GEM-NE was the largest, while Blank-NE had the smallest particles. It is noteworthy to mention that the z-average diameter of the combination formulation, 5DOX/5GEM-NE, was significantly lower than that of the single agent formulation, 5DOX-NE and 5GEM-NE. In contrast, there were no significant differences between the entire NE formulations in the small values of PDIs and the negative charges of the zeta potentials.

MTT Assay for Cytotoxicity Evaluation of Drug Formulas Against Various Cancer Cells

Not all the tested drug formulations had a considerable antiproliferative effect on the A549 and HCT116 cells. In terms of the drug formulations effect on the HeLa cells (Figure 1), it has been found that both 5DOX-NE and 5DOX/5GEM-NE have the best inhibitory effect on impeding the proliferation of the HeLa cells as the cell viabilities were 30 ± 3.21 and 27.00 ± 5.62, respectively. Interestingly, combining 5GEM with 5DOX in distilled water has significantly improved the cytotoxic effect of both GEM and DOX as the cell viabilities of 5DOX-D.W, 5GEM-D.W and 5DOX/5GEM-D.W were 89.87 ± 4.50, 87.00 ± 4.29 and 77.91 ± 0.24, respectively. In addition, loading GEM in NE has considerably enhanced the antiproliferative effect of GEM as the cell viabilities of 5GEM-D.W and 5GEM-NE were 87.00 ± 4.29 and 76.42 ± 3.48, respectively. Similarly, the inhibitory effect of the 5DOX-NE formula was greater than 5DOX-D.W by three fold.

Furthermore, HFS cells were administered into the drug formulas in order to examine their adverse effects on the healthy cells (Figure 1). Interestingly, all of the NE formulations were less toxic than the water formulations. In addition, the combination formula 5DOX/5GEM-NE was the least toxic (cell viability = 97.06 ± 1.09) among all formulations.

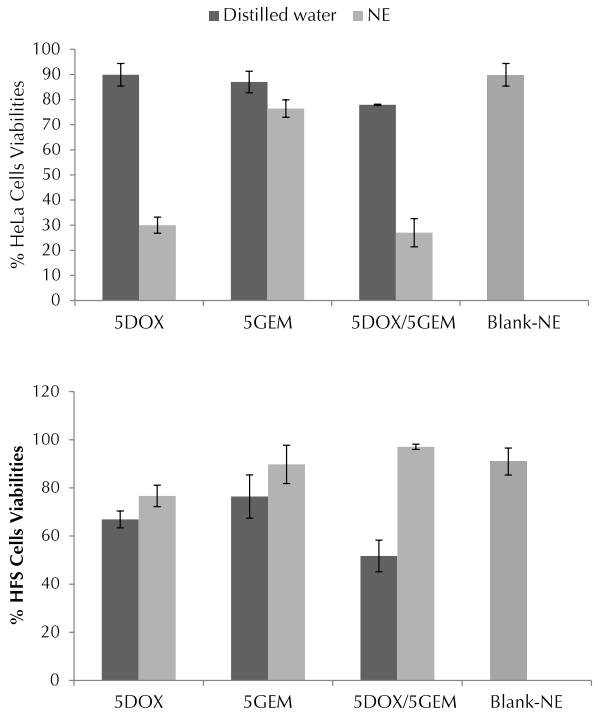

Figure 1. The Cell Viabilities of the HeLa Cervical Cancer Cells and HFS Human Foreskin Cells Subjected to the Drug Formulations for 24 Hours.

Table 1. The Z-average Diameters, PDIs and Zeta Potential Measurements of NE Formulations

Formulations	Z-Average Diameter (nm)	PDI	Zeta Potential (mV)
Blank-NE	136.00 ± 12.47	0.09 ± 0.36	-7.95 ± 1.30
5DOX-NE	199.20 ± 8.13***	0.04 ± 0.32	-7.16 ± 4.76
5GEM-NE	255.93 ± 9.90***	0.04 ± 0.30	-6.33 ± 1.20
5DOX/5GEM-NE	155.38 ± 3.08*	0.02 ± 0.28	-7.70 ± 1.30

* There is a significant difference between the Blank-NE and the desired drug-loaded formula; ***There is a very highly significant difference between the Blank-NE and the desired drug-loaded formula

Data were expressed as mean ± SD.

Apoptotic Effect of the Drug Formulas

Light Microscopy

The detected signs of apoptosis include membrane blebbing, chromatin condensation, intercellular spaces and cellular shrinkages. Figure 2 exhibits the effect of the NE formulations on the HeLa cells. Cells treated with Blank-NE did not differ from the untreated cells, whereas cells treated with 5DOX-NE and 5DOX/5GEM-NE have displayed increased intercellular spaces, cellular shrinkages, membrane blebbing and chromatin condensation. In contrast, 5GEM-NE have shown limited cytotoxicity in the cells since most of the cells appeared with condensed chromatin.

Regarding the effect of the NE formulations on HFS cells, it was revealed that cells treated with Blank-NE did not differ from the untreated cells (Figure 2). On the other hand, cells treated with 5DOX-NE showed an increase in the intercellular spaces and membrane blebbing unlike the cells treated with 5GEM-NE, which showed a slight increase in the intercellular spaces. Cells treated with 5DOX/5GEM-NE displayed a limited chromatin condensation and increased intercellular spaces.

Nuclear Staining Using DAPI

The blue-fluorescent, DAPI, stains dsDNA when associated with A-T clusters in the minor groove. The decrease in the intensity of the fluorescent stains of the A-T base pair indicates alteration of the DNA that leads to the degradation of the cells. Figure 2 displays the effect of the NE formulations on the HeLa and HFS cells while the histogram in Figure 2 exhibits the percentages of fluorescent intensities of the treated cells. Among the studied formulations, 5DOX-NE has the greatest effect on the DNA of both HeLa and HFS cells as the least fluorescent intensities were observed in the treated cells. In contrast, Blank-NE has the least cytotoxic effect on the HFS and HeLa cells. Interestingly, 5DOX/5GEM-NE has a great cytotoxic effect on the nucleus of the HeLa cells but very limited side effect on the HFS cells.

ApopNexin™ FITC Apoptosis Detection Assay

FITIC/PI Double staining method was used to distinguish between necrotic (Q1), late apoptotic (Q2), viable (Q3) and early apoptotic (Q4) cells. The apoptotic effects of NE formulations on HeLa and HFS cells were demonstrated

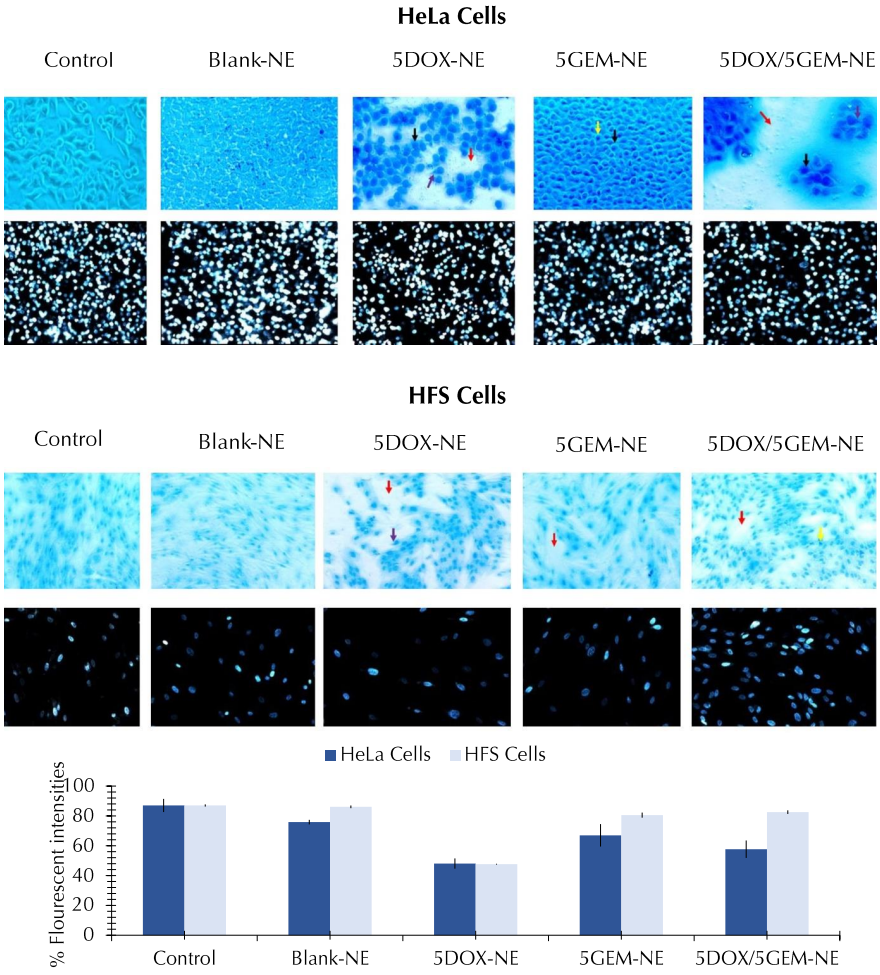

Figure 2. Light microscopy and DAPI fluorescent images of the cells treated with the drug formulations for 24 hours. Images were magnified at 20X. Signs of apoptosis were represented by the purple arrows (membrane blebbing), yellow arrows (chromatin condensation), red arrows (intercellular space).

in flow cytometry plots while the percentages of apoptotic cells (Q_2 + Q_4) were displayed in Figure 3. It has been found that both 5DOX-NE and 5DOX/5GEM-NE have a comparable apoptotic effect on the HeLa cells as the percentages of apoptotic cells were the largest among all treated cells. In contrast, cells treated with Blank-NE has the smallest percentage of apoptotic cells. On the other hand, 5DOX/5GEM-NE has a very limited apoptotic effect on the HFS cells when compared to the single treatments, 5DOX-NE and 5GEM-NE.

Discussion

In the present study, the cytotoxicity screening of both NE and solution formulations was assessed in the HeLa, A549, HCT116 and HFS cells by MTT assay. Results revealed that none of the NE formulations have a cytotoxic effect on A549 and HCT116 cells. In addition, not all the solution formulations had a considerable cytotoxic effect on the HeLa cells, whereas the NE formulations have a great antiproliferative effect. In particular, 5DOX-NE and 5GEM-NE showed a better antitumor activity unlike 5DOX-D.W and 5GEM-D.W, which proved that NE formula enhanced the activity of the drugs. The study of Amjad et al (14) demonstrated that the improved cytotoxicity of DOX-encapsulated in micelles is attributed to the increased permeability and retention of micelles in cancer cells. According to the study by Du et al (15), encapsulating DOX into lipid NEs increased the antitumor activities by 1.6 fold, which was attributed to the faster internalization of DOX into cells mediated by NE. The results of a study by Jaidev et al (16) showed that encapsulation of the GEM inside poly (lactic-coglycolic acid) (PLGA) nanospheres was more effective than the use of free drug in the treatment of pancreatic cancer. The study of Trickler et al (17) found that GEM released from the nanoparticles has improved the drug bioavailability inside the cell.

In this study, 5DOX-NE had a greater toxic effect than 5GEM-NE and 5DOX/5GEM-NE. However, the combination of DOX and GEM has improved the efficacy of GEM and reduced the side effect of DOX. In agreement with our study, Liu et al (18) have shown that DOX-loaded micelles were much more effective than GEM-loaded micelles against the same cancer cells with the same drug incubation time. In addition, the results of Wang et al (19) demonstrated that the combinational use of pacilataxel (PTX) and DOX (M(PTX/Dox)) loaded in micelles against MCF7 and HepG2 cells are more toxic than the

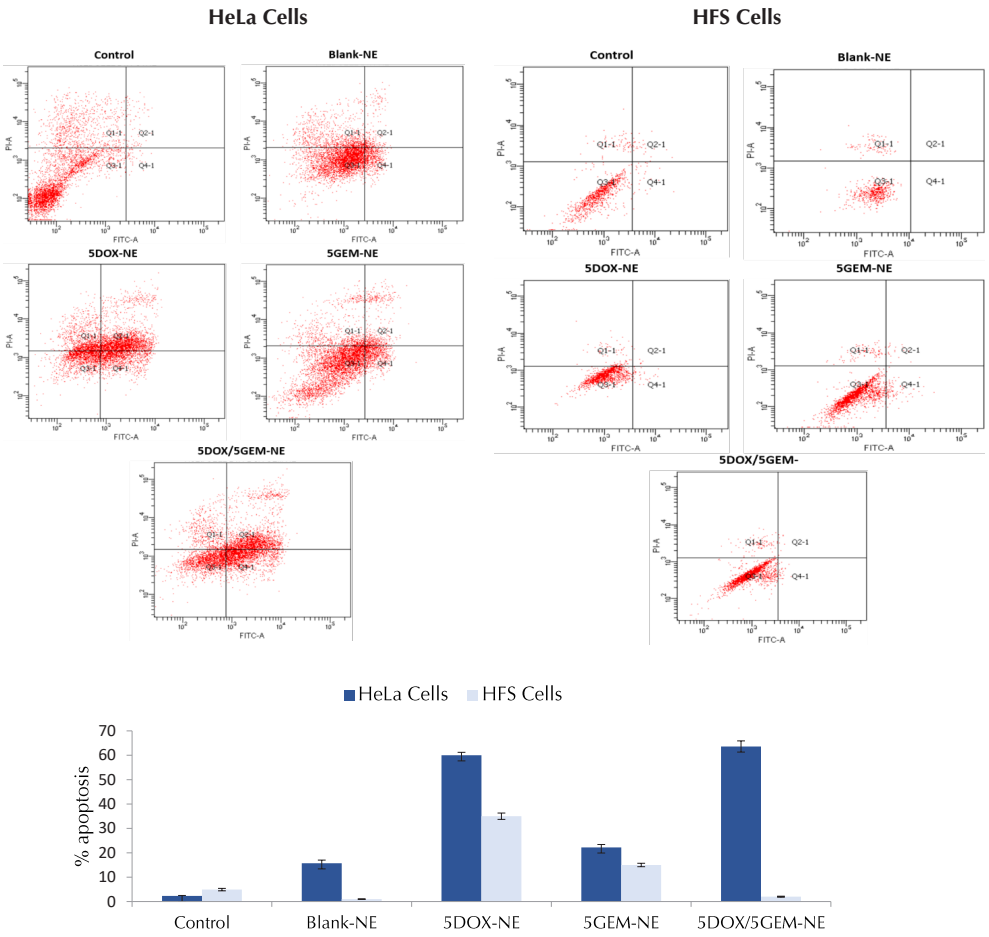

Figure 3. FITC/PI flow cytometry plots and histogram presentation of the apoptosis percentage (Q_2+Q_4) of the HeLa cervical cancer cells and HFS human foreskin cells treated with NE formulations for 24 hours.

single drug use which is due to the synergistic effect of the combinational use of PTX/Dox and M(PTX/Dox).

Interestingly, drugs-loaded NE had a less cytotoxic effect against HFS cells than drugs-loaded solutions. 5DOX/5GEM-NE had the least cytotoxic effect which was significantly less than 5DOX/5GEM-D.W indicating that it has less side effects on the healthy cells. In a study done by Asadishad et al (20), it has been reported that free DOX was more toxic to healthy cells (HFF cells) than the DOX-loaded GdNPs (gold nanoparticles), which indicates that the nanocarriers have eliminated the adverse effect of DOX on the healthy cells.

Within 24 hours, different levels of apoptosis were observed when the selected NE formulations were used. All drug-loaded NE formulations have exhibited an apoptotic effect against HeLa cells. Loading the drugs into nanoparticles improved their accumulation in the nuclei and thereby stimulate the apoptotic effect (12,21). It could be due to the small size of the nanoparticles that enhance the permeation of the drugs into the cells without rapid degradation (22). As it appears in the results of the current study, 5GEM-NE has the larger size while when it was combined with 5DOX-NE, the particle size decreased. In addition, all of the NE formulations have a negative surface charge (zeta potential) which implies that the nanoparticles in NE permeated the cells by binding the cationic sites on the surface of the cell membrane (23).

Conclusions

According to the physical characterization of NE formulations, it has been found that loading GEM and DOX on NE resulted in producing small negatively charged nanodroplets with z-average diameter of 155.38 ± 3.08 nm and low PDIs (<1.00), which indicate a uniform and homogeneous distribution of the nanodroplets. Neither the single treatment of DOX and GEM nor their combination, loaded on NE, have had cytotoxicity in A549 and HCT116 cells. However, these NE formulations have induced apoptosis in the HeLa cells. The combination of DOX and GEM in NE has shown a very limited adverse effect on the HFS cells when compared to the single treatments of DOX and GEM.

Ethical Issues

The ethical approval was obtained from the Research Ethics Committee in the Faculty of Medicine at King Abdulaziz University.

Conflicts of Interests

Authors declare that they have no conflict of interests.

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