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Evaluation of the *in vivo* genotoxicity of liposomal formulation for delivering anticancer estrogenic derivative (ESC8) in a mouse model

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ABSTRACT

The genotoxic potential of glucocorticoid receptor (GR)-targeted liposomal formulations of the anticancer drug molecule ESC8 was studied *in vivo*. A methodical literature review discovered no previous studies on the genotoxicity of ESC8. Genotoxicity was assessed in both male and female mice by various assay systems, such as comet assay, chromosomal aberrations and micronuclei assay, which detect different abnormalities. Eleven groups of male mice and eleven groups of female mice, containing six animals per group, were used in the present study: group I served as vehicle control; group II received the positive control (cyclophosphamide 40 mg/kg; CYP); and animals in group III to XI received free drug (ESC8), DX liposome and drug-associated DX liposomal formulation (DXE), respectively, dissolved in 5% solution of glucose at a drug-dose of 1.83, 3.67 and 7.34 mg/kg, respectively. Same drug treatments were followed for the female mice groups. The obtained data revealed the safety of DXE, which did not show substantial genotoxic effects at different dose levels. In contrast, the positive control, CYP, exhibited highly substantial irregular cytogenetic variations in comparison with the control group in different assays.

1. Introduction

Testing for the ability to induce mutations is the primary phase in the assortment of chemicals for progress and a necessary component of governing approval and marketing, whether it is an industrial chemical, pesticide, drug or a food additive (Ames et al., 1973a; Ames et al., 1973b). Genotoxicity is a broad term, which points to the potential undesirable effects of a substance on genetic material not necessarily linked with mutagenicity. Therefore, in addition to the tests for mutagenicity, the tests for genotoxicity serve as an indication of the damage induced to deoxyribonucleic acid (DNA) through various effects such as DNA strand breaks, unscheduled DNA synthesis (UDS), formation of DNA adducts, sister chromatid exchange (SCE) and mitotic recombination (Helleday et al., 2008; Purchase et al., 1978). Chemotherapy agents usually possess a low therapeutic index i.e., while they target the cancer cells, the surrounding normal cells are also affected and hence adversely affect cancer patients, which strictly limits their use. Besides, some research studies have evaluated the hazards to employees handling anti-cancer drugs (Gulten et al., 2011; Kopjar et al., 2009). The health hazards for medical workers handling these drugs is a key concern equally, since these drugs are categorized as potentially mutagenic, carcinogenic or teratogenic (Turci et al., 2002). Exposure occurs primarily to the hands but occasionally to other parts of the body as well. As these substances directly or indirectly affect the DNA, medical workers exposed to these drugs have higher chances of suffering DNA damage (Deng et al., 2005; Rombaldi et al., 2009).

Current melanoma therapies are incompetent of killing cancer stem cells, which represents a substantial break in functionality. We have previously studied antigen-negative variant-1 (ANV-1), a breast cancer stem cell (CSC)-like cell line with mesenchymal characteristics that has undergone an epithelial-to-mesenchymal transition (EMT) (Santisteban et al., 2009). As this mouse line is highly drug resistant and shows tremendous aggressiveness in

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mouse models (Santisteban et al., 2009), we have selected it for use in the current study. Recently, our group also developed an anti-breast cancer molecule, ESC8, that showed expression of ER independent toxicity in breast cancer cells through the concurrent induction of autophagy and apoptosis (Sinha et al., 2011), but exhibited remarkably low toxicity to non-breast cancer cells. The expression of Neuropilin-1 (NRP-1) in ANV-1 cells is at least four-fold higher than parent mouse meningeal cells (MMC) cells with epithelial character. The transcription factor, Id-1, is also upregulated, which maintains the characteristics of CSC through the inhibition of the MET transition (data not shown). We hypothesized that ANV-1 would be a decent model to examine the special effects of NRP-1 on CSC-like cells as we found the downregulation of NRP-1 caused cells to partially revert to the epithelial character (de-differentiation) and reduced aggressiveness during tumor formation (data not shown). We also found that ANV-1 was more resistant to our anti-breast cancer drug, ESC8, at concentrations in which other breast cancer cells with no CSC-like properties were highly sensitive. Hence, targeting CSC-like cells will require further drug discovery work. The goal of the present study was to examine the genotoxic potential of the lipid-derived formulation of the anti-cancer agent DXE, using the chromosomal aberration and the comet and micronucleus assays. Additionally, the other main components in this formulation (i.e., the drug, ESC8) were individually tested and compared for their genotoxic effects (if any).

2. Materials and methods

2.1. Chemicals and reagents

Synthesized ESC8 and lipid based formulation of ESC8 (DXE) were obtained from CSIR-IICT, Hyderabad, India. High quality purified water was produced in our laboratory by Millipore water purification system. Additional reagents, chemicals and organic solvents were acquired from either Rankem Ltd. (Mumbai, India) or Sigma Inc. (St. Louis, MO, USA).

2.2. Preparation of liposomes

Liposomes were prepared following the method of Mukherjee et al. (2009). The DX liposomes contained DODEAC: Chol: Dex in 1:1:0.75 mol ratios, where DODEAC means N,N-dioctadecyl N,N-dihydroxyethyl ammonium chloride; Chol means cholesterol and Dex means dexamethasone. For the entrapment of ESC8 drug (hydrophobic) in DX liposome, chloroform solution of ESC8 was added to the chloroform solution of lipids' mixture of DX at 0.25 M ratios with respect to DODEAC lipid. The organic solvent was evaporated, dried under vacuum and then hydrated with 5% glucose overnight. The liposomes (DX and DXE) were first bath sonicated and then probe sonicated (Branson, CT, USA). The formulations were used as such with no further removal of any unentrapped drug. The purities of ESC8 and DODEAC used in this study were >99%. Other formulation components of DX, including dexamethasone and cholesterol so procured from commercial vendors were also of >99% purity.

2.3. Animal welfare provision and animal care

Male and female albino mice Balb/C57 (weight: 25–30 g; age: 8–12 weeks old), were acquired from the Animal House and Care Center in College of Pharmacy, King Saud University. All mice were kept under normal conditions of humidity, temperature (25 ± 2 °C), and light (12-h light/dark), with free access to water and served a normal mice pellet diet. All experimental procedures in the study were conducted in accordance with the established standards of humanitarian animal care as described in the procedures of NIH and Committee for Prevention, Control, and Supervision of Animal Experiments of the Kingdom of Saudi Arabia.

2.4. Experimental protocol

The study was conducted as per the protocol and mice were divided into twenty-two groups, with six males and six female mice in each group (n = 6), including a vehicle-control and positive control groups. Experiments were carried out to evaluate the genotoxicity and bone marrow cytotoxicity of ESC8, DX and DXE (the liposomal formulation of ESC8) *in vivo*. The animals were intraperitoneally administered 5% glucose solution as a vehicle (Group I), 40 mg/kg cyclophosphamide as a positive control (group II), ECS8, DX, and DXE were dispersed in 5% glucose, respectively, at 1.83, 3.67 and 7.34 mg/kg (groups III, IV, V, VI, VII, VIII, IX, X and XI). The female mice in each group received the same treatment as male mice groups. After administration, the animals were observed for 24 h for signs of toxicity and death. The animals were sacrificed 24 h after treatment by cervical dislocation.

2.5. Genotoxicity tests

2.5.1. Comet assay

The slides for each animal were prepared within 1 h after sacrifice (Sharma et al., 2009; Singh et al., 1988). Mice were sacrificed and bone marrow cells from one femur were collected in tubes containing ice-cold PBS. Each cell suspension was shaken mildly to mix the bone marrow cells and kept back on ice for 20–30 s to allow the cells to settle before use. Small amount of the supernatant was diluted with a fresh aliquot of low melting point 0.5% agarose dissolved in Dulbecco's phosphate buffer at 37 °C and placed on microscopic slides pre-coated with (1%) normal melting agarose. Agarose (0.5%; *w/v*) was used as a top layer over the gel-embedded cells and enclosed with a cover slip for 5–10 min at 4 °C. To allow DNA unwinding, gel fixed cells were lysed in a lysing solution (2.5 M NaCl, 10 mM Trizma base, 100 mM disodium EDTA, 8 g/L NaOH, pH 10 using NaOH) at 4 °C for 20 min (Marques et al., 2016; Nandhakumar et al., 2011). The samples were electrophoresed for 20 min at 50 V and 400 mA. The slides were stained with 20 mg/mL ethidium bromide (EtBr) and observed under a microscope (Fluorescence, Olympus IX41) armed with a BP5 10 nm excitation filter and a 590 nm barrier filter. The slides were examined by a computerized imaging system and the amount of DNA damage was evaluated by the obtained tail moment values. For each slide one hundred cells were scored. The DNA damage was evaluated by using the following parameters: head DNA, tail DNA, tail moment and tail migration.

2.5.2. Micronucleus test (MN test)

Normochromatic and polychromatic erythrocytes (NCEs and PCEs) were evaluated in the MN test (Attia et al., 2009). The mice were sacrificed 24 h after treatment and both the femurs were removed from each animal. The slides of bone marrow smears were prepared as follows; exposed femurs were cut to make openings on both ends of the bone and the bone marrow was extracted by using a syringe filled with fetal bovine serum. This cell suspension was shifted to a centrifuge tube, filled with 2–5 ml heat-inactivated fetal calf serum (Gibco, USA), and centrifuged for 10 min at 1000 rpm. The supernatant was aspirated and the cells were re-suspended in fetal bovine serum. A small droplet from this suspension was spread on a clean sterilized microscopic slide. Two smears of bone marrow cells were arranged for each animal, air-dried, and then fixed in methanol. The smears were subsequently labelled and stained with May-Grünwald Giemsa (ID, 1984). From each mouse, 1000 PCEs and 1000 NCEs were observed for micro

nucleated erythrocytes (MNPCEs and MNNCEs) at ×1000 magnification by using a microscope. Furthermore, the number of PCEs among 1000 NCEs per mice was noted to estimate bone marrow suppression and the ratio of PCE/NCE was calculated from the formula: %PCE = [PCE/(PCE + NCE)] × 100.

2.5.3. Chromosome aberration analysis

The same drug administration schedule was followed as the micronucleus and comet assay tests. Six mice per group were intraperitoneally injected with 4 mg/kg colchicine 90 min before sacrifice to arrest the cell division. The slides for chromosome analysis were arranged and stained as described by Attia (2008), Attia et al. (2009). In brief, both femurs were dissected and bone marrow was flushed from the femurs in foetal calf serum (FCS). The bone marrow cells were dispersed gently by pipetting and collected by centrifugation at 1100 rpm; the harvested cells were incubated in 10 ml of 0.075 M KCl for 20 min at 37 °C. After incubation, Carnoy's fixative (cold glacial acetic acid–methanol, 1:3, v/v) was added to each tube and again centrifuged for 10 min at 1100 rpm. The supernatant was discarded and 10 ml of fresh fixative was gently pipetted onto the cells without disturbing the pellet. Two to three changes of fixative were done before the preparation of slides. Finally, the cells were suspended in a small volume of fixative and burst open on a clean slide to release chromosomes. The slides were air dried and stained with 5% Giemsa and coded before observation. All the slides were labelled and scored at ×1000 magnification under a microscope (Nikon). For each animal, one hundred metaphase plates (for each group 600 metaphases) were counted for both numerical and structural aberrations in cells of bone marrow. The cells were categorized into five categories as per the severity of damage: cells having fragments, gaps, breaks only, rings and Sister chromatid exchange (SCE). The cells containing gaps were not involved in the calculation of total chromosomal aberrations owing to their disruptive genetic significance. The same slides were used to evaluate the cells for their mitotic activity. The mitotic index of cells was calculated from the portion of dividing cells out of a populace of 1000 cells.

2.6. Statistical analysis

The data obtained was analyzed and presented as a frequency percentage and the Student-Newman-Keuls test was used to check the significance using a primer software. Values of $p < 0.05$ were considered to fulfil the significance criterion.

3. Results

The bone marrow cells of animals were evaluated for DNA damage after treatment with the test compounds. Table 1 represents the results of comet assay, which indicated no substantial induction of breaks in DNA strands in any of the treatment groups. A significant escalation in DNA damage was noticed in animals treated with ESC8 higher doses, as indicated by the scores obtained. In contrast, the positive control group treated with CYP displayed substantial rise in DNA damage in comparison to the vehicle control and treatment groups.

Aneugenic/clastogenic damage was examined by the analysis of formation of micronuclei in the bone marrow of PCEs. The micronuclei assay of the bone marrow cells in the DX and DXE groups revealed that they did not significantly alter the percentage of PCEs and MNPCEs in contrast with the control; however, 40 mg/kg of CYP, used as positive control in the micronucleus

Table 1
DNA damage in mice after the treatment with ESC8, DX, and DXE, as described in the Methods. *,# Denote significant differences from the control and positive control groups, respectively (p < 0.05). (A), (B) and (C) denotes different doses as 1.83, 3.67, and 7.34 mg/kg.

Treatment groups	*Male mice (n = 6)*				*Female mice (n = 6)*			
	Head DNA	Tail DNA	Tail moment	Migration	Head DNA	Tail DNA	Tail moment	Migration
Normal	96.71 ± 1.04	3.29 ± 1.04	0.57 ± 0.27	1.31 ± 0.39	96.49 ± 1.13	3.51 ± 1.13	0.75 ± 0.29	1.33 ± 0.27
CYP	76.23 ± 1.02*	23.72 ± 1.18*	6.68 ± 0.70*	14.32 ± 1.61*	74.98 ± 1.68*	24.88 ± 1.02#	7.02 ± 0.58#	16.05 ± 1.25#
ESC8 (A)	92.74*# ±2.04*#	7.26 ± 0.99*#	0.31 ± 0.11#	2.00 ± 0.81*#	92.22 ± 2.65*#	7.85 ± 1.03*#	0.38 ± 0.09*#	1.89 ± 0.75*#
(B)	90.99 ± 1.11*#	9.11 ± 1.09*#	0.56 ± 0.26#	2.65 ± 0.36*#	90.71 ± 0.87*#	9.29 ± 0.89*#	0.34 ± 0.14*#	2.29 ± 0.15*#
(C)	86.05 ± 2.91*#	13.94 ± 1.27*#	0.81 ± 0.24*#	2.50 ± 0.91*#	87.45 ± 2.91*#	12.56 ± 1.27*#	0.62 ± 0.32#	2.00 ± 0.69*#
DX (A)	96.05 ± 1.89#	3.94 ± 1.12#	0.28 ± 0.01*#	1.51 ± 0.41*#	96.05 ± 1.89*#	3.94 ± 1.12#	0.28 ± 0.01*#	1.51 ± 0.41#
(B)	95.83 ± 1.17#	4.16 ± 1.82*#	0.35 ± 0.16#	1.75 ± 0.35*#	95.39 ± 0.50#	4.61 ± 0.52*#	0.28 ± 0.16*#	1.79 ± 0.76*#
(C)	90.93*# ±1.88*#	9.06 ± 1.32*#	0.49 ± 0.21#	1.00 ± 0.08*#	90.93 ± 1.88*#	9.06 ± 1.32*#	0.49 ± 0.21*#	1.00 ± 0.08#
DXE (A)	95.93*# ± 1.81#	4.07 ± 1.09#	0.41 ± 0.29#	1.56 ± 0.12*#	95.93 ± 1.81#	4.07 ± 1.09#	0.41 ± 0.29*#	1.56 ± 0.12#
(B)	94.57 ± 1.02*#	5.42 ± 0.99*#	0.51 ± 0.18#	1.68 ± 0.43*#	94.73 ± 0.52#	5.16 ± 0.53*#	0.50 ± 0.19*#	1.72 ± 0.20#
(C)	90.99 ± 2.08*#	9.00 ± 1.22*#	0.59 ± 0.24#	2.51 ± 0.68*#	90.99 ± 2.08*#	9.00 ± 1.22*#	0.59 ± 0.24*#	2.51 ± 0.68#

Table 2
Frequencies of MNPCEs and PCEs in bone marrow of mice treated with ESC8, DX, and DXE, as described in the Methods. *, # Denote significant differences from the control and positive control groups, respectively (p < 0.05). (A), (B) and (C) denotes different doses as 1.83, 3.67, and 7.34 mg/kg.

Treatment groups	*Male mice (n = 6)*		*Female mice (n = 6)*	
	% MNPCE	% PCE	% MNPCE	% PCE
Normal	0.45 ± 1.06	49.82 ± 6.27	0.45 ± 0.57	49.91 ± 3.52
CYP	1.41 ± 0.58*	40.91 ± 3.05*	1.37 ± 0.56*	40.02 ± 2.70*
ESC8 (A)	0.53 ± 0.20*#	45.56 ± 2.69*#	0.52 ± 0.18*#	45.88 ± 3.26
(B)	0.61 ± 0.60*#	44.89 ± 3.16*#	0.58 ± 0.88*#	45.51*# ± 6.53
(C)	1.05 ± 0.76*#	42.56 ± 4.85*#	1.03 ± 0.82*#	42.98 ± 3.51*
DX (A)	0.49 ± 0.12*#	47.18 ± 3.57#	0.50 ± 0.19*#	47.65 ± 3.21*#
(B)	0.48 ± 0.76#	48.94 ± 7.87#	0.51 ± 0.47*#	48.74 ± 5.03*#
(C)	0.50 ± 0.18*#	49.12 ± 4.28*#	0.51 ± 0.22*#	49.66 ± 3.83*#
DXE (A)	0.48 ± 0.22#	46.01 ± 3.59*#	0.49 ± 0.20*#	46.49 ± 4.10*#
(B)	0.51 ± 0.70*#	46.89 ± 5.34*#	0.53 ± 0.76*#	47.04 ± 7.31*#
(C)	0.78 ± 0.31*#	45.46 ± 3.44*#	0.74 ± 0.36*#	45.81 ± 3.81*#

assay, exhibited a significant increase in the PCE/MNPCEs ratio (Table 2). As expected, exposure to higher dose of ESC8 led to a substantial escalation in the number of micronucleated cells in comparison with the vehicle control and DXE formulation. The bone marrow cell cytotoxicity was measured through the determination of the PCE/NCE ratio, and it was shown that DXE did not reduce the PCE/NCE ratio when equated with the positive control. The vehicle group of mice exhibited a few irregular metaphases and aberrations per hundred metaphases. The CYP-treated animals exhibited many instances of an abnormal metaphase and a statistically important number of chromosomal anomalies in comparison with those shown by the vehicle group. The occurrence of chromosomal irregularities in other treatment groups comprised mostly of fragments, gaps, ring formation, and sister chromatid exchanges, whereas the CYP treated group contained approximately 50% abnormalities in total (Table 3).

4. Discussion

Liposome-encapsulated anti-cancer drugs have emerged as a valuable method for the delivery of chemotherapeutic agents (Papagiannaros et al., 2006), reducing their non-specific toxicity and enhancing their anti-cancer effect (McLoon and Wirtschafter, 1999). ESC8, a predominantly lipophilic molecule, remains attached to the lipophilic core of DX liposome, enabling DX to simultaneously deliver both pDNA and drugs. DX confers cancer cell selectivity and access to the GRE promoter, which may permit the use of other lipophilic anti-cancer agents or functional compounds within the DX formulation to probe the tumor microenvironment. This drug-associated DX formulation (DXE) eradicated highly aggressive and drug-resistant ANV-1 cells, whereas treatment with either unencapsulated ESC8 or NRP-1 shRNA in a DX-lipoplex did not; this was putatively attributable to targeting of the GRE promoter. This targeting also allows the cargo to evade the cytoplasmic mechanisms of drug resistance. The anticancer effect, which was evident in 100% of cells, occurred within 3 h of treatment and exceeded the transfection efficiency of 20–25% attained in conventional cationic liposome strategies.

Cyclophosphamide (CYP) is a well-known alkylating agent and is often used as an anticancer agent for the treatment of several malignancies (Colvin, 1999). The compound produces a carbonium ion that reacts with DNA and proteins to induce lethal mutations and mononuclei and to generate ROS and cause cellular damage (Arif et al., 2009). The most lethal effects of the CYP free radicals *in vivo* are genotoxic activities, including DNA mutilation, chromosomal abnormalities, sister chromatid exchanges, and gene mutations, which lead to a number of pathological conditions, including cancer (Anderson et al., 1995). Several studies have reported that intraperitoneal administration of CYP can lead to DNA damage, chromosomal abnormalities and sister chromatid exchanges, as well as a reduction in mitotic index (Martins et al., 1998; Murata et al., 2004).

In the present investigation, we have compared the genotoxicity of a conventional drug, CYP, and a liposomal DX-based formulation of ESC8 (DXE). The data obtained clearly indicated the induction of significant DNA damage to CYP-treated bone marrow (Pfuhler and Wolf, 1996) in comparison with unencapsulated free-drug ESC8 and the liposomal formulation-associated drug, i.e., DXE. There have been studies that have discovered that formulations are often more toxic than their active constituents. Therefore, it is imperative to consider the genotoxic and cytotoxic effects exerted by these active components in the drug risk assessment (Lin and Garry, 2000; Zeljezic et al., 2006). The excessive production of free radicals (e.g. ROS/RNS) induces severe damage of DNA, as indicated by the lymphocyte comet assay (Ghosh et al., 2012). The positive control,

Table 3
Frequencies of different types of chromosomal aberrations (CA) and mitotic activity in bone marrow of mice 24 h after treatment with ESC8, DX, and DXE. $^{*\#}$ Denote significant differences from the control and positive control groups ($p < 0.05$). (A), (B) and (C) denotes different doses as 1.83, 3.67, and 7.34 mg/kg.

	Normal	CYP	ESC8 (A)	ESC8 (B)	ESC8 (C)	DX (A)	DX (B)	DX (C)	DXE (A)	DXE (B)	DXE (C)
Male mice (n = 6)											
Normal	92.80 ± 0.48	51.83 ± 2.00*	87.29 ± 1.69$^{*\#}$	84.54 ± 0.72$^{*\#}$	78.39 ± 1.88$^{*\#}$	92.74 ± 2.35$^{*\#}$	91.69 ± 0.56$^{\#}$	90.54 ± 1.26$^{\#}$	90.49 ± 1.29$^{\#}$	89.83 ± 0.49$^{*\#}$	85.91 ± 1.21$^{*\#}$
Fragments	2.04 ± 0.14	12.68 ± 0.72*	4.00 ± 0.91*	4.36 ± 0.28*	8.39 ± 0.69*	2.26 ± 0.21$^{\#}$	2.53 ± 0.48$^{*\#}$	2.46 ± 0.18$^{*\#}$	3.00 ± 0.21$^{*\#}$	2.73 ± 0.68$^{*\#}$	7.00 ± 0.53$^{*\#}$
GAP	1.40 ± 0.20	11.20 ± 0.86*	3.25 ± 0.64$^{*\#}$	3.20 ± 0.20$^{*\#}$	6.00 ± 0.56$^{*\#}$	2.00 ± 0.31$^{*\#}$	1.99 ± 0.13$^{*\#}$	2.00 ± 0.09$^{*\#}$	2.30 ± 0.18$^{*\#}$	3.05 ± 0.36$^{*\#}$	3.02 ± 0.12$^{*\#}$
SCE	1.71 ± 0.23	20.88 ± 0.87*	3.25 ± 0.49$^{*\#}$	4.60 ± 0.19$^{*\#}$	5.10 ± 0.39$^{*\#}$	1.10 ± 0.09$^{*\#}$	1.35 ± 0.10$^{\#}$	1.5 ± 0.11$^{\#}$	2.11 ± 0.09$^{*\#}$	2.52 ± 0.23$^{*\#}$	2.05 ± 0.09$^{*\#}$
Break	–	–	–	–	–	–	–	–	–	–	–
Ring	2.05 ± 0.04	3.42 ± 0.30*	2.21 ± 0.12$^{\#}$	3.30 ± 0.11$^{*\#}$	2.12 ± 0.16$^{\#}$	1.00 ± 0.02$^{*\#}$	2.44 ± 0.16$^{*\#}$	2.5 ± 0.25$^{*\#}$	2.09 ± 0.11$^{\#}$	1.88 ± 0.34$^{\#}$	2.00 ± 0.11$^{\#}$
TA	7.20 ± 0.18	48.17 ± 4.13	12.71 ± 0.98	15.46 ± 0.42	21.61 ± 0.92	7.36 ± 0.69	8.31 ± 0.31	8.46 ± 0.85	9.51 ± 0.21	10.17 ± 0.29	14.09 ± 0.86
Female mice (n = 6)											
Normal	92.20 ± 0.46	52.07 ± 0.91*	87.29 ± 1.69$^{*\#}$	85.40 ± 1.03$^{*\#}$	78.39 ± 1.88$^{*\#}$	92.74 ± 2.35$^{*\#}$	91.80 ± 1.07$^{*\#}$	90.54 ± 1.26$^{*\#}$	90.49 ± 1.29$^{*\#}$	90.00 ± 1.14$^{*\#}$	85.91 ± 1.21$^{*\#}$
Fragments	2.00 ± 0.10	13.72 ± 1.09*	4.00 ± 0.91$^{*\#}$	3.60 ± 0.24$^{*\#}$	8.39 ± 0.69$^{*\#}$	2.26 ± 0.21$^{\#}$	1.60 ± 0.24$^{*\#}$	2.46 ± 0.18$^{*\#}$	3.00 ± 0.21$^{*\#}$	2.40 ± 0.24$^{*\#}$	7.00 ± 0.53$^{*\#}$
GAP	2.00 ± 0.14	11.20 ± 0.86*	3.25 ± 0.64$^{*\#}$	3.20 ± 0.20$^{*\#}$	6.00 ± 0.56$^{*\#}$	2.00 ± 0.31$^{\#}$	2.20 ± 0.37$^{\#}$	2.00 ± 0.09$^{\#}$	2.30 ± 0.18$^{*\#}$	2.60 ± 0.40$^{*\#}$	3.02 ± 0.12$^{*\#}$
SCE	2.20 ± 0.15	19.85 ± 0.69*	3.25 ± 0.49$^{*\#}$	4.20 ± 0.25$^{*\#}$	5.10 ± 0.39$^{*\#}$	1.10 ± 0.09$^{*\#}$	2.00 ± 0.14$^{\#}$	1.5 ± 0.11$^{*\#}$	2.11 ± 0.09$^{\#}$	2.66 ± 0.21$^{*\#}$	2.05 ± 0.09$^{\#}$
Break	–	–	–	–	–	–	–	–	–	–	–
Ring	1.60 ± 0.24	3.16 ± 0.06*	2.21 ± 0.12$^{*\#}$	3.60 ± 0.19$^{*\#}$	2.12 ± 0.16$^{*\#}$	1.00 ± 0.02$^{*\#}$	2.40 ± 0.17$^{*\#}$	2.5 ± 0.25$^{*\#}$	2.09 ± 0.11$^{*\#}$	2.40 ± 0.24$^{*\#}$	2.00 ± 0.11$^{*\#}$
TA	7.80 ± 0.15	47.93 ± 3.99	12.71 ± 0.98	14.60 ± 0.24	21.61 ± 0.92	7.36 ± 0.69	8.20 ± 0.20	8.46 ± 0.85	9.51 ± 0.21	10.06 ± 0.08	14.09 ± 0.86

SCE: Sister chromatid exchange; TA: Total aberration.

CYP, induces breaks in the DNA strand, DNA synthesis termination, protein-DNA crosslinks, and DNA adduct formation, which eventually lead to genotoxicity (Yuksel et al., 2017). In this study, the proportion of damaged bone marrow cells in mice showed a significant escalation of the comet tail in CYP-treated mice in comparison with normal mice. There was a substantial reduction in DNA damage and comet tail in the following order: CYP > ESC8 > DX > DXE > vehicle control. Our data clearly illustrates that DXE exhibited significantly lower toxicity than positive control CYP. Based on these results, another genotoxicity assay was carried-out to compare the number of chromosomal aberrations in the bone marrow of the treated mice and the control mice. Chromosomal aberrations (CA) are alterations in the arrangement of chromosome that result from a disruption or altercation of chromosomal material. Generally, CAs detected in cells are harmful, but there are several anomalies that do not impair cell viability, and instead genetic effects are inherited (Swierenga et al., 1991). The data indicated that no induction of chromosomes or chromatid aberrations in metaphasic arrest were found after treatment with ESC8, DX, and DXE, but the positive control, CYP, induced a statistically significant number of structural anomalies, with regard to the fragmentation, GAP, SCE, break, ring, and TA in bone marrow cells. The mutagenic potential for each group was found to be in the order CYP > DXE > DX > ESC8 > normal.

MN assays have been frequently used for the evaluation of genotoxicity since 1970 (Heddle, 1973; Matter and Schmid, 1971). The frequency of micronuclei is contingent upon the rate of chromosomal rupture and CA or the damage and frequency of cell division (von Ledebur and Schmid, 1973). Micronuclei are present in virtually all dividing cells. Mice bone marrow and lymphocytes were used for the micronucleus test and the PCE ratio was used as the guideline for cytotoxicity. The study was considered only when all PCE ratios were larger than 0.20 (Heddle, 1973; von Ledebur and Schmid, 1973). Clastogenic/aneugenic damage was studied through the analysis of micronuclei formation in bone marrow PCEs. The test treatments did not tempt any substantial rise in the micronucleus incidence in the bone marrow cells, neither was a significant difference/increase observed in the PCE/NCE ratio in the DX-, ESC8-, and DXE-treated cells. The order of the clastogenic/aneugenic potential was as follows: CYP > ESC8 > DX > DXE > vehicle control. These experiments have demonstrated that CYP is a potent bone marrow clastogen when administered to animals (Cole et al., 1979; Yuksel et al., 2017). Therefore, it is likely that exposure to CYP led to a substantial rise in the quantity of micronucleated cells in contrast to the vehicle group. Bone marrow cell cytotoxicity was determined through the quantification of the PCE/NCE ratio, which indicated that DXE did not decline the PCE/NCE ratio in comparison with the vehicle control. This study therefore demonstrates for the first time that, at low doses, DXE is neither genotoxic nor cytotoxic *in vivo,* in mouse bone marrow cells and in PCE cells. Moreover, the DXE formulation sheltered the mouse bone marrow cells against the CYP-induced genetic damage and reduction in the cell proliferation, noticed by a decline in DNA damage, micronuclei frequency and chromosomal aberrations. CYP, the positive control, was used as a mutagen to ensure the expected responses were achieved from the experimental set up; the results of the treatments with this drug are in corroboration with previous studies (Ashby and Beije, 1985; Jenderny et al., 1988; Thust, 1982). Thus, these results established the understanding of the experimental protocol for the uncovering of genotoxic effects.

5. Conclusion

Our findings suggested that the single intraperitoneal administration of different doses of the liposomal formulation of ESC8 (DXE) did not show a significant degree of genotoxicity in leukocytes and the bone-marrow cells of mice. The micronucleus test demonstrated that DXE exerted no clastogenic or aneugenic effects on the bone marrow as well. As compared with results obtained for the un-encapsulated ESC8 versus DXE, ESC8 produced mild DNA damage and chromosomal aberration in mice.

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Conflict of interest

The authors declare that there is no conflict of interest.

Compliance with Ethical Standards

All experimental procedures in the study were conducted in accordance with the accepted standards of humanitarian animal care as described in the guidelines of NIH and of Committee for Prevention, Control, and Supervision of Animal Experiments of the Kingdom of Saudi Arabia.

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