Safety assessment of the pharmacological excipient, diethylene glycol monoethyl ether (DEGEE), using in vitro and in vivo systems

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Abstract

Background Diethylene glycol monoethyl ether (DEGEE) is widely used as a solubilizer in cosmetics as well as in oral, topical, transdermal and injectable pharmaceutical formulations. Due to the unavailability of detailed toxicological studies on DEGEE, the Scientific Committee on Consumer Products (SCCP) found its toxicological reports to be unsatisfactory, comprising only summaries. Also, a few reports have raised concern on the use of DEGEE as it might cause damage to the kidneys.

Objective Safety assessment of DEGEE using in vitro and in vivo models.

Methods In vitro effects of DEGEE (0.5–25 mg/ml) were assessed in the HEK293 human embryonic kidney cells. In vivo effects were evaluated after single acute exposure of DEGEE via intraperitoneal route in *Swiss albino* mice and further, a 28 days subchronic exposure study was conducted where DEGEE was administered orally, once daily.

Results DEGEE was cytotoxic to HEK293 cells, and an IC_{50} of 15 mg/ml was established. An increase in the intracellular levels of ROS and alteration in the mitochondrial membrane potential led to nuclear fragmentation and induction of apoptosis in these cells. Survival rate of animals administered intraperitoneally with a single acute dose of 1000 mg/kg DEGEE was 100% with no significant changes in the behavioural and histological parameters. However, the dose of 3000 mg/kg and above led to total mortality within 14 days of acute exposure. Subchronic oral exposure of 500–2000 mg/kg DEGEE showed no significant changes in the hematological, biochemical and histopathological parameters.

Conclusions The in vitro findings indicate that the nephrotoxic potential of DEGEE cannot be ruled out. The results of the in vivo studies reveal that the degree of toxic effects shown by DEGEE varies, depending on the dose, duration of exposure and routes of administration. Therefore, the present findings are of relevance and thorough studies should be conducted before using this substance in clinical formulations.

Keywords Excipient · Diethylene glycol monoethyl ether · Safety assessment · Acute exposure · Subchronic exposure

Abbreviations

DEG	Diethylene Glycol
DEGEE	Diethylene Glycol Monoethyl Ether
SRB	Sulforhodamine B Assay
FACS	Fluorescence-Activated Cell Sorting
ROS	Reactive Oxygen Species
H ₂ DCFDA	2',7'-dichlorodihydrofluorescein Diacetate
MMP	Mitochondrial Membrane Potential

ALT	Alanine Aminotransferase
AST	Aspartate Aminotransferase
ALP	Alkaline Phosphatase
T-BIL	Total Bilirubin
BUN	Blood Urea Nitrogen

Introduction

Excipients are therapeutically inactive components in a drug formulation mostly used to enhance the stability, penetration or bioavailability of the active principle [1]. The safety of excipients came under scrutiny in the year 1937, when 76 humans died, and several became ill with kidney failure after

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consumption of toxicologically untested Diethylene glycol (DEG) that was added to sulfanilamide antibacterial drug by the manufacturers [2]. Further, DEG adulterated formulations claimed the life of 47 children from Nigeria (1990), 30 children from Haiti (1996) and 33 children from India (1998) [3–5]. In the ensuing years, guidelines were developed for the safety testing of excipients earlier by the Safety committee of the International Pharmaceutical Excipients Council (1996) and more recently, the US Food and Drug Administration/ Centre for Drug Evaluation and Research published the guidance entitled “Nonclinical Studies for the Safety Evaluation of Pharmaceutical Excipients” [6]. Thorough risk assessment becomes an integral part of the drug development process to ensure the safety of newly discovered excipients.

Diethylene glycol monoethyl ether (DEGEE), an ethylene oxide derivative, is used as a solvent in numerous products in the pharmaceutical, cosmetics and food industry. It has application as an effective solubilizer in oral, topical, transdermal and injectable human and veterinary pharmaceutical products. It is noteworthy that the industrial grades of this solvent contain significant levels of ethylene glycol and DEG impurities. A recent review by Dexter W. Sullivan Jr. et al. suggested that the toxicity of DEGEE reported in earlier studies can be attributed to the presence of these impurities [7].

Based on the available scientific literature, The Scientific Committee on Consumer Products (SCCP) conducted a risk assessment of DEGEE in 2006, which led one of the member states to put some restrictions on the use of this substance. The available ‘Toxicological reports’ were found unsatisfactory by the SCCP as they contained only summaries without details [8]. An isolated case report cited that the accidental ingestion of approximately 300 ml of a liquid containing 47% DEGEE (approx. 2000 mg/kg) by a male led to the occurrence of severe symptoms of central nervous, respiratory injury, thirst, acidosis and albumin in the urine. The subject recovered following symptomatic treatment. Although in 2013, the SCCP revised the systemic daily dose of DEGEE in cosmetic products for dermal application, its use in products for oral hygiene and the eyes were not evaluated [9]. Reports from India highlighted that the use of 99.8% DEGEE in the painkiller injectables might cause damage to the kidneys, therefore, a thorough safety assessment of DEGEE becomes crucial [10]. Preliminary in vitro findings have also indicated that DEGEE was able to inhibit the growth of Neuro2A neuroblastoma cell line in dose as well as time-dependent manner [11].

Considering the above reports and lack of scientific conclusions [12], studies were performed in Human embryonic kidneys cells, HEK 293 to evaluate the effect of DEGEE exposure and further its safety assessment was carried out in *Swiss albino* mice. Ninety-nine percent pure form of DEGEE was used to rule out any possibility of impurity associated toxic effects.

Material and methods

Reagents

DEGEE (537616), cell culture medium DMEM high glucose, gentamicin (G3632), sulforhodamine B (SRB) (S1402), trichloroacetic acid (T6399) were purchased from Sigma-Aldrich Co. (Sigma, St. Louis, MO, USA). Fetal Bovine Serum (16,000,044), penicillin-streptomycin antibiotics (15,140,122), 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) (D-399) and JC-1 dye (T3168), were obtained from Gibco, Thermo Fisher Scientific (Grand Island, NY, USA). Annexin V FITC/PI apoptosis kit (556547) was purchased from BD Bioscience (San Jose, CA, USA).

Ames assay

Salmonella typhimurium tester strains, TA98, TA97a, TA100, and TA102, were purchased from MOLTOK, USA. The bacterial concentration of each strain was maintained above 10^8 cells/ml after overnight culture. Hepatic microsomal enzymes (S9) were induced from rat liver homogenates by Aroclor1254, and the concentration of S9 mixture was 10%. DEGEE doses used in the experiment were 10, 33, 100, 333, and 1000 µg/dish. Positive control drugs used were 2.5 µg/dish of 4-nitro-o-phenylenediamine, 50 µg/dish of 9-Aminoacridine, 5 µg/dish of Sodium azide, 0.5 µg/dish of Mitomycin C and 5 µg/dish of 2-Aminoanthracene. Solvent group (double-distilled sterile water) was set at 0.1 mL/dish. The plates were cultured for 48 h at 37 °C, and the number of revertant colonies was counted.

Cell lines and culture

Human embryonic kidney epithelial cell line, HEK 293, was procured from National Centre for Cell Sciences (NCCS, Pune, India). Cells were cultured in DMEM high glucose medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin solution and 50 µg/ml gentamicin. Cells were maintained in 5% CO₂ humidified incubator at 37 °C.

Cell viability assay

Cytotoxic effect of DEGEE was assessed on HEK 293 cells by the SRB assay as described earlier [13]. Briefly, cells were plated in 96 well plate and incubated at 37 °C for the next 24 h. Cells were treated with 0.5 mg/ml – 25 mg/ml DEGEE for 12 h and 24 h post which they were fixed with 10% trichloroacetic acid and stained with SRB. Subsequently, protein-bound SRB was dissolved in 10 mM Tris Base, and absorbance was recorded at 510 nm by a microplate reader (BIOTEK).

Annexin V-FITC assay for apoptosis

DEGEE induced apoptosis was studied by staining cells with FITC conjugated Annexin V/Propidium Iodide (PI) [14]. Briefly, HEK 293 cells were seeded in 6 well plate at a density of 2×10^5 cells per well and treated with 7.5 mg/ml – 15 mg/ml DEGEE for 24 h. Later, cells were harvested by trypsinization and labelled with FITC tagged Annexin V and PI as per manufacturer's protocol (BD Bioscience). Cells were analysed using BD FACS Calibur flow cytometer.

Examination of chromatin condensation

DEGEE induced chromatin condensation was visualised in the cells following the Hoechst staining protocol [15]. Briefly, 2×10^5 cells were seeded in a six well plate and treated with 7.5 mg/ml – 15 mg/ml DEGEE for 24 h. Following the treatment, cells were washed and fixed with 3.7% paraformaldehyde (pH 7.4) and permeabilized with 0.2% Triton X-100. Permeabilized cells were stained with 1 µg/ml Hoechst- 33,258 and fragmented nuclei were examined in an inverted fluorescence microscope (EVOS, Life Technologies).

Estimation of reactive oxygen species (ROS)

Considering ROS generation to be an early event, the cells were seeded in 6 well plate and treated with 7.5 mg/ml – 15 mg/ml DEGEE for 6 h – 12 h. Cells were harvested by trypsinization and subsequently incubated with 10 µM H₂DCFDA and analysed in a flow cytometer post 6 h of treatment. For microscopic examination, cells were treated with DEGEE for 12 h and washed with 1X PBS (Phosphate buffer saline) and stained with 10 µM H₂DCFDA. Cells were incubated for 30 min at 37 °C in the dark after which they were washed twice with PBS and visualised at 400X magnification in a fluorescence microscope (EVOS, Life Technologies).

Detection of alteration in mitochondrial membrane potential (MMP; $\Delta\Psi_m$)

HEK 293 cells were plated in 6 well plate and treated with 15 mg/ml DEGEE to assess the time dependent alteration in MMP from 3h – 12 h. For assessment of dose dependent effect on MMP, cells were treated with 7.5 mg/ml – 15 mg/ml DEGEE for 6 h. Cells were harvested and incubated with 2 µg/ml JC-1 dye for 30 min at 37 °C in the dark and were washed with PBS. Cell acquisition and analysis was performed on a flow cytometer.

Toxicity evaluation in mice

Experimental animals

Swiss albino mice of either sex (8 weeks of age), weighing 20–25 g were obtained from Laboratory Animal Division of Central Drug Research Institute, India following Institutional Animals Ethics Committee clearance (IAEC/2016/2). The animals were housed under standard environmental conditions (temperature, 25 °C; humidity, 51% ± 10%) with a 12 h light–dark cycle and were fed a standard pelleted diet and water ad libitum. The animals were allowed to acclimatize to laboratory conditions for a week before starting the experiment and were randomly allocated to four groups containing six animals each (three males and three females). All procedures performed in studies involving animals were in accordance with the ethical standards of the institution at which the studies were conducted.

Acute exposure

An acute single dose intraperitoneal (i.p) exposure of DEGEE prepared in sterile water was administered at doses 1000 mg/kg, 3000 mg/kg, and 5000 mg/kg to the mice. The control mice received only 100 µl sterile water. The body weight, food and water consumption, and mortality were recorded daily for 14 days. At the end of the experiment, animals were sacrificed by cervical dislocation, and their organs were collected for histological analysis.

Subchronic exposure

For a subchronic repeated dose, DEGEE prepared in sterile water was orally administered once daily for 28 days in single doses of 500 mg/kg, 1000 mg/kg, and 2000 mg/kg. The control mice received only 200 µl sterile water. The body weight, food and water consumption, signs of illness, changes in physical appearance, behavioural pattern and mortality were recorded daily throughout the study period. Blood was collected at the termination of the experiment for further analysis post which the animals were sacrificed, and their organs were collected to assess histological changes.

Hematological and biochemical analysis The following hematological parameters were analysed using an automatic hematology analyzer (Sysmex-XT-2000i, Germany): white blood cells (WBCs), red blood cells (RBCs), hemoglobin concentration (Hb), platelet count and hematocrit.

The following serum biochemical parameters were measured using a biochemistry autoanalyzer (Vital Scientific, Junior Series Clinical Chemistry Analyzer, The Netherlands): urea, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin (T-BIL), creatinine and blood urea nitrogen (BUN).

Histological analysis The liver, kidneys, heart, brain, spleen, and lungs were excised and weighed using an analytical balance (Sartorius BT-124S, Germany). These organs were then fixed in 10% buffered neutral formalin for histological examination post-sectioning and hematoxylin and eosin staining. The grading of the histological examination was categorized from minimal, mild, moderate to severe.

Statistical analysis

All the graphs and data were analysed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA. One-way ANOVA was used to measure statistical significance between control and treated groups and $p < 0.05$ were considered as statistically significant.

Results

Effects of DEGEE on the mutagenicity of standard strains in vitro

Ames toxicity results are shown in Table 1. The mean spontaneous revertant colonies of all the four strains were within the normal allowable range with or without S9 conditions. The positive control 4-nitro-o-phenylenediamine, 9-Aminoacridine, sodium azide, mitomycin C and 2-aminoanthracene showed significant differences ($p < 0.001$), and produced more revertant colonies than the negative control on strains TA98, TA97a, TA100 and TA102. Whereas, the DEGEE test groups resulted in a lower number of revertant colonies showing no statistical difference ($p > 0.05$) in doses of 10, 33, 100, 333, and 1000 $\mu\text{g}/\text{dish}$. The results indicated that no mutagenicity effects were produced in the four standard *Salmonella typhimurium* strains by tested doses of DEGEE under the experimental conditions.

Cytotoxic effect of DEGEE in HEK 293 cells

To investigate the effect of DEGEE on kidney cell viability, human embryonic kidney cells, HEK 293, were exposed to increasing doses of DEGEE ranging from 0.5 mg/ml to 25 mg/ml. DEGEE showed no inhibitory effect between the treatment concentrations of 0.5 mg/ml – 2.5 mg/ml but proceeded towards cytotoxicity at 5 mg/ml post 24 h of treatment. The cytotoxic response of DEGEE was found to be dose and time-dependent (Fig. 1a). Further analysis of these results yielded inhibitory concentration 50 (IC50) values for DEGEE to be 15 mg/ml.

Effect of DEGEE on induction of apoptosis and chromatin condensation in HEK 293 cells

The effect of DEGEE on the induction of apoptosis in HEK 293 cells was analyzed by Annexin V/PI staining using flow cytometric approach. As shown in Fig. 1b, c, when compared to untreated HEK 293 cells carrying 7.63% of Annexin V/PI positive cells, the fraction of cells undergoing apoptosis increased post 24 h of DEGEE exposure, in a dose-dependent manner, with 24.89%, 49.3% and 52.94% apoptotic cells at doses 7.5 mg/ml, 10 mg/ml and 15 mg/ml respectively ($P < 0.001$).

Chromatin condensation and apoptotic nuclear morphology were observed by fluorescent dye Hoechst 33258. Untreated HEK 293 cells appeared faintly blue and had intact nuclear morphology whereas, brightly stained fragmented nuclei were observed in DEGEE exposed cells. Maximum DNA damage was observed at the highest dose of DEGEE used, i.e., 15 mg/ml (Fig. 1d).

Effect of DEGEE exposure on intracellular ROS generation

To assess the involvement of ROS in DEGEE mediated cytotoxicity in HEK 293 cells, the cells were stained with

Table 1 Results of the Ames test conducted with DEGEE (0–1000 $\mu\text{g}/\text{plate}$) for three independent experiments

	TA98		TA97a		TA100		TA102	
	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9
Solvent control	32 ± 1	27 ± 8	171 ± 32	170 ± 5	225 ± 28	62 ± 5	175 ± 4	121 ± 2
10 $\mu\text{g}/\text{plate}$	33 ± 1	25 ± 6	216 ± 25	182 ± 6	203 ± 12	47 ± 8	166 ± 9	122 ± 13
33 $\mu\text{g}/\text{plate}$	35 ± 2	22 ± 3	205 ± 16	167 ± 12	183.5 ± 1	62 ± 4	173 ± 14	128 ± 4
100 $\mu\text{g}/\text{plate}$	37 ± 4	15 ± 2	201 ± 13	168 ± 16	176 ± 23	60 ± 1	182 ± 10	122 ± 28
333 $\mu\text{g}/\text{plate}$	27 ± 4	17 ± 3	197 ± 11	157 ± 10	212 ± 12	54 ± 11	165 ± 5	131 ± 6
1000 $\mu\text{g}/\text{plate}$	31 ± 4	17 ± 1	193 ± 1	157 ± 14	178 ± 2	61 ± 4	166 ± 5	123 ± 2
Positive controls	1281 ± 36***	880 ± 96***	1104 ± 264***	622 ± 33***	1092 ± 107***	997 ± 15***	907 ± 55***	485 ± 10***

Double distilled sterile water was used as negative control (solvent). Data shown as mean ± standard deviation (SD) revertants/plate for three replicates for each concentration in each experiment. *** $P < 0.0001$ highly significant compared to controls

Fig. 1 Effect of DEGREE on cell proliferation, apoptosis and chromatin condensation. **a** DEGREE inhibited cell proliferation of human embryonic kidney cells, HEK 293 at 12 h and 24 h time points. **b, c** HEK 293 cells were treated with 7.5 mg/ml – 15 mg/ml of DEGREE for 24 h. Apoptosis was analysed by Annexin V/PI staining and cells were acquired via flow cytometry. Data was statistically analysed. **d** DEGREE

induced chromatin condensation and altered nuclear morphology was examined in HEK 293 cells by Hoechst staining using fluorescence microscope at 200x magnification; Scale bar = 200 μ m. Results were expressed as mean $\pm$ SD ($n = 3$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, calculated compared to control

the cell-permeant non-fluorescent 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) dye which gets converted to fluorescent dichlorofluorescein (DCF) by ROS and analyzed by flow cytometer. Post 6 h of DEGREE exposure, when compared to the control cells, the intracellular ROS levels increased dose-dependently by 2 folds at an exposure of 7.5 mg/ml. However, at the highest tested dose of 15 mg/ml, a 6 fold increase in the ROS levels were observed (Fig. 2a, c). The cells were also visualized under a fluorescence microscope for validation, and an increase in fluorescence intensity was observed in the DEGREE treated cells (Fig. 2b).

Effect of DEGREE exposure on alteration of MMP

Alteration in MMP was examined in HEK 293 cells using JC-1 dye which exists as a green-fluorescent monomer at depolarized membrane potentials. However, in hyperpolarized cells, the dye forms J-aggregates and produces red fluorescence. Untreated cells were observed with high red:green fluorescence whereas, 15 mg/ml DEGREE promoted membrane depolarization which was found to be highest post 6 h of the treatment but decreased at 12 h (Fig. 3a, b). Further, the 6 h time point was chosen, where the J-monomers increased dose-dependently with a 2 fold increase observed in

Fig. 2 Effect of DEGEE on the levels of reactive oxygen species (a) HEK 293 cells were treated with 7.5 mg/ml – 15 mg/ml of DEGEE for 6 h. ROS levels were analysed by H₂DCFDA staining and were acquired via flow cytometer. (b) Generation of ROS in HEK 293 cells treated with 7.5 mg/ml – 15 mg/ml of DEGEE for 12 h was confirmed using

fluorescence microscope at 400x magnification; Scale bar = 200 μ m. (c) Statistical analysis of DCFDA positive HEK 293 cells treated with DEGEE as compared to control. Results were expressed as mean $\pm$ SD (n = 3). **P < 0.01, ***P < 0.001, calculated compared to control

the HEK 293 cells treated with 15 mg/ml DEGEE when compared to the untreated cells (Fig. 3c, d).

Effect of acute exposure to DEGEE on Swiss albino mice

The changes in body weight, as well as the food and water intake of the animals during the observation period of 14 days, postsingle exposure of 1000 mg/kg DEGEE were calculated and have been listed in Table 2. At day 14, the gain in mean body weight (b.w.) of male mice administered with 1000 mg/kg was 2.67 ± 0.74 g and that in female mice was 1.50 ± 0.32 g which were similar to the mean b.w. gain of control male mice (1.87 ± 2.61 g) and control female mice (4.46 ± 0.82 g). No changes in the food and water consumption pattern were observed. Histological analysis of the kidney, liver, heart and

lungs revealed no significant changes as compared to the control group (Fig. 4b). However, the survival of animals significantly declined at higher doses where all the animals treated with 3000 mg/kg died by day 9. Thirty three percent mortality was observed within 24 h in the group treated with the highest tested dose of DEGEE, i.e. 5000 mg/kg and mortality rate reached hundred percent by day 6, post treatment (Fig. 4a).

Effect of subchronic exposure to DEGEE on Swiss albino mice

Effect of DEGEE on body weight and food and water intake

DEGEE administered orally at doses 500, 1000 and 2000 mg/kg, daily for 28 days did not result in any mortality in the test animals. No general behavioural changes and signs

Fig. 3 Effect of DEGREE on the alteration of mitochondrial membrane potentials (MMP) (a–b) Time dependent effect on change in MMP in HEK 293 cells treated with 15 mg/ml of DEGREE. (c–d) Dose dependent effect on change in MMP in HEK 293 cells treated with

7.5 mg/ml - 15 mg/ml of DEGREE for 6 h. Depolarized MMP was detected by JC-1 staining and was acquired via flow cytometry. Results were expressed as mean $\pm$ SD (n = 3). **P < 0.01, ***P < 0.001, calculated compared to control

of toxicity were observed during the experimental duration. The body weight, as well as the food and water intake of the animals, were calculated and recorded in Table 3. At day 28, the mean b.w. of control male mice increased from 23.93 ± 1.81 g to 32.60 ± 2.98 g (mean b.w. gain, 8.67 ± 1.17 g) and

that of control female mice increased from 23.50 ± 0.50 g to 30.06 ± 1.50 g (mean b.w. gain, 6.56 ± 1.00 g) which were similar to the mean b.w. gain of mice treated with the highest dose, i.e., 2000 mg/kg of DEGREE (7.74 ± 0.22 g in males; 6.07 ± 1.94 g in females). The food consumption of male mice receiving 2000 mg/kg DEGREE was decreased by 42.08% ($P < 0.001$) whereas the volume of water consumed was increased by 58.42% ($P < 0.01$) (Fig. 5a, b). The females of the same group (2000 mg/kg) showed a decrease in food and water intake by 36.9% and 35.87% ($P < 0.001$) respectively, in comparison to the control group by the 28th day of the study (Fig. 5d, e).

Table 2 Effect of single intraperitoneal administration of 1000 mg/kg DEGREE on the body weight and food & water intake of Swiss albino mice

	Control		1000 mg/kg	
	Male	Female	Male	Female
Body Weight (grams)				
Pre-treatment	30.33 $\pm$ 2.12	24.27 $\pm$ 0.50	29.73 $\pm$ 2.44	23.97 $\pm$ 1.33
Day 1	30.35 $\pm$ 2.24	24.74 $\pm$ 0.23	29.33 $\pm$ 1.53	23.80 $\pm$ 1.11
Day 7	33.07 $\pm$ 3.25	27.20 $\pm$ 1.25	32.27 $\pm$ 0.70	25.80 $\pm$ 1.22
Day 14	32.20 $\pm$ 4.73	28.73 $\pm$ 2.32	32.40 $\pm$ 3.18	25.47 $\pm$ 1.01
Food Intake (grams)				
Pre-treatment	5.49 $\pm$ 0.59	5.09 $\pm$ 0.10	4.41 $\pm$ 0.85	4.79 $\pm$ 0.26
Day 1	5.15 $\pm$ 0.04	4.83 $\pm$ 0.12	4.05 $\pm$ 0.34	3.46 $\pm$ 0.51
Day 7	4.96 $\pm$ 0.49	4.35 $\pm$ 0.12	4.84 $\pm$ 1.15	4.38 $\pm$ 0.21
Day 14	4.51 $\pm$ 0.66	4.12 $\pm$ 0.32	5.34 $\pm$ 0.54	3.82 $\pm$ 0.15
Water Intake (ml)				
Pre-treatment	6.67 $\pm$ 0.46	5.09 $\pm$ 0.11	6.54 $\pm$ 0.54	4.31 $\pm$ 0.24
Day 1	7.27 $\pm$ 0.72	4.89 $\pm$ 0.22	8.07 $\pm$ 0.17	5.93 $\pm$ 0.28
Day 7	7.45 $\pm$ 0.32	5.43 $\pm$ 0.61	7.12 $\pm$ 0.42	5.05 $\pm$ 0.23
Day 14	6.76 $\pm$ 1.03	4.31 $\pm$ 0.35	5.34 $\pm$ 0.54	4.33 $\pm$ 0.17

All values are expressed as mean $\pm$ S.D

Effect of DEGREE on the mean organ weights

The mean organ weights of the control and treated mice were recorded (Table 4). No significant differences were observed in the weight of the vital organs of treated and control groups of either sex except for the male mice receiving 1000 mg/kg DEGREE, where the mean weight of the liver was increased significantly by 17.7% ($P < 0.01$) (Fig. 5c, f).

Effect of DEGREE on hematological and biochemical parameters

The effects of subchronic treatment on the hematological and biochemical parameters are presented in Table 3. All the tested hematological parameters, including total WBCs and total RBCs count, haemoglobin, platelet count and hematocrit,

Fig. 4 Effect of acute intraperitoneal administration of DEGEE on *Swiss albino* mice (a) The survival curve of animals treated with 1000, 3000, or 5000 mg/kg DEGEE as compared to control group. (b) The

histological analysis of the liver, kidney, heart and lungs of mice post 14 days of treatment with 1000 mg/kg DEGEE or the vehicle. Images were acquired at 200x magnification; Scale bar = 200 μ m

were within the normal range. Also, no significant changes in the liver and renal function test parameters, i.e. urea, ALT, AST, ALP, T-BIL, creatinine and BUN were recorded after the oral administration of DEGEE for up to 28 days.

Effect of DEGEE on the histological parameters

The histological examination provided evidence of minimal hydropic degeneration of the hepatocytes in almost all groups of animals. Minimal focal to minimal multifocal areas of mononuclear cells (MNCs) were observed in the liver of two out of six animals administered with 500 mg/kg DEGEE and in one animal in the highest dose group (2000 mg/kg) however, similar changes were observed in two out of six animals in the control group as well. Areas of minimal focal MNCs were observed in the kidneys of DEGEE treated groups, and minimal dilation of the Bowman's capsule was observed in the kidney of two females in the high dose group. The kidneys of all other animals were normal. No histopathological abnormalities were detected in the spleen, lungs, brain or heart of the control group or the treated groups (Fig. 6).

Discussion

The emerging use of DEGEE as an excipient in various formulations necessitates its thorough safety evaluation [16–19]. The pharmaceutical and cosmetic industries, as well as the regulatory bodies, emphasize on the routine use of in vitro methods for drug screening, safety assessment and risk evaluation as these cellular models can mimic the in vivo physiological processes and allow better prediction of an outcome in vivo [20]. Therefore, HEK 293, a human embryonic kidney cell line, was selected in this study for investigation of specific cellular events and end points [21].

In the present study, the cytotoxic effects of DEGEE on HEK 293 cells have been demonstrated in a time and dose dependent manner. The results of this study showed that treatment of HEK 293 cells with DEGEE led to an increase in the level of intracellular ROS. The mitochondria serve as one of the major sources of intracellular ROS generation, however, excessive production of ROS can lead to disruption of its membrane potential [22]. It has been demonstrated in this study, that DEGEE induced alterations in MMP in a time

Table 3 Effect of oral administration of 500–2000 mg/kg DEGEE for 28 days on the body weight, food & water intake, haematological and biochemical parameters of *Swiss albino* mice

	Control		500 mg/kg		1000 mg/kg		2000 mg/kg	
	Male	Female	Male	Female	Male	Female	Male	Female
Clinical observations	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Body Weight (grams)								
Pre-treatment	23.93 ± 1.81	23.50 ± 0.50	25.20 ± 2.83	22.20 ± 1.20	24.93 ± 1.33	22.00 ± 2.36	26.13 ± 1.50	18.53 ± 3.05
Day 1	25.20 ± 2.11	24.27 ± 0.23	26.20 ± 2.09	22.73 ± 1.28	26.86 ± 1.22	21.73 ± 2.61	27.73 ± 3.05	19.33 ± 2.66
Day 7	28.80 ± 1.11	25.80 ± 0.60	26.33 ± 5.68	22.33 ± 3.70	30.60 ± 0.35	23.60 ± 2.23	28.13 ± 2.80	20.20 ± 2.69
Day 14	32.80 ± 0.53	28.07 ± 1.79	30.67 ± 4.40	24.27 ± 3.67	34.93 ± 1.14	26.20 ± 1.22	28.00 ± 0.60	22.53 ± 2.40
Day 21	30.80 ± 3.14	29.20 ± 1.25	33.53 ± 3.87	26.07 ± 4.01	35.13 ± 4.35	28.6 ± 2.70	30.73 ± 1.86	24.27 ± 2.08
Day 28	32.60 ± 2.98	30.06 ± 1.50	36.73 ± 3.91	28.20 ± 2.80	35.80 ± 4.16	28.2 ± 3.22	33.87 ± 1.72	24.60 ± 1.11
Food Intake (grams)								
Pre-treatment	6.94 ± 0.52	4.93 ± 0.11	6.30 ± 0.71	4.44 ± 0.24	7.98 ± 0.43	4.18 ± 0.45	7.06 ± 0.41	4.26 ± 0.70
Day 1	6.55 ± 0.55	4.37 ± 0.04	5.50 ± 0.44	3.63 ± 0.21	6.45 ± 0.29	5.08 ± 0.73	5.27 ± 0.30	6.90 ± 1.10
Day 7	6.62 ± 0.26	4.40 ± 0.10	5.80 ± 1.25	3.79 ± 0.63	7.04 ± 0.08	4.01 ± 0.38	5.50 ± 0.45	4.04 ± 0.54
Day 14	6.72 ± 0.11	6.84 ± 1.07	7.17 ± 2.46	6.98 ± 2.57	6.76 ± 0.54	4.03 ± 0.79	6.36 ± 0.37	5.44 ± 1.75
Day 21	7.16 ± 1.45	6.26 ± 0.52	6.73 ± 1.35	6.07 ± 2.16	7.05 ± 1.74	5.15 ± 1.24	5.21 ± 1.04	5.34 ± 1.14
Day 28	8.34 ± 1.31	7.23 ± 0.66	7.97 ± 0.74	6.25 ± 0.62	6.52 ± 1.16	5.84 ± 0.35	4.83 ± 0.55	4.56 ± 0.52
Water Intake (ml)								
Pre-treatment	8.38 ± 0.63	8.21 ± 0.17	8.32 ± 0.93	8.42 ± 0.44	8.73 ± 0.47	7.4 ± 0.47	8.89 ± 0.51	7.26 ± 0.70
Day 1	6.55 ± 0.55	8.37 ± 0.04	5.50 ± 0.44	7.41 ± 0.19	6.45 ± 0.29	5.91 ± 0.47	5.27 ± 0.30	5.22 ± 0.72
Day 7	8.35 ± 0.32	7.48 ± 0.17	8.16 ± 1.76	7.25 ± 1.03	7.04 ± 0.80	6.61 ± 0.62	7.31 ± 0.73	5.05 ± 0.67
Day 14	8.48 ± 0.18	8.61 ± 0.36	8.28 ± 1.19	6.55 ± 1.01	9.83 ± 0.35	8.38 ± 0.39	10.08 ± 0.22	6.34 ± 0.89
Day 21	9.32 ± 1.25	8.43 ± 0.57	9.09 ± 1.74	8.68 ± 2.56	10.11 ± 1.87	9.20 ± 1.24	13.06 ± 0.91	5.04 ± 1.29
Day 28	9.91 ± 1.37	10.23 ± 0.66	10.08 ± 2.77	10.25 ± 1.32	11.50 ± 3.08	8.84 ± 1.35	15.70 ± 1.10	6.56 ± 0.52
Haematology								
WBC ($\times 10^3$ / μ l)	7.94 ± 0.46	8.54 ± 0.38	7.22 ± 1.04	7.48 ± 0.82	7.02 ± 1.22	6.8 ± 1.24	4.56 ± 0.64	5.20 ± 1.30
RBC ($\times 10^6$ / μ l)	11.26 ± 1.72	8.66 ± 2.04	10.46 ± 1.34	8.94 ± 1.06	9.84 ± 2.16	8.91 ± 1.48	9.34 ± 2.08	9.22 ± 1.68
Hb (g/dl)	16.40 ± 1.30	12.90 ± 2.24	14.80 ± 0.91	13.10 ± 1.42	13.9 ± 2.06	12.40 ± 1.85	13.50 ± 0.94	12.60 ± 0.47
Platelet ($\times 10^3$ / μ l)	665 ± 12	586 ± 36	642 ± 28	584 ± 39	614 ± 13	602 ± 23	686 ± 17	512 ± 42
Haematocrit (%)	50.0 ± 0.8	44.5 ± 1.3	45.9 ± 2.8	48.8 ± 0.6	47.4 ± 0.9	40.9 ± 2.1	45.6 ± 1.8	43.4 ± 1.6
Serum Biochemistry								
Urea (mg/dl)	50.7 ± 3.3	41.8 ± 2.1	49.9 ± 3.2	49.6 ± 3.2	46.9 ± 2.6	43.1 ± 3.2	52.1 ± 1.4	45.2 ± 2.4
ALT (U/L)	165.9 ± 9.4	136.2 ± 6.0	162.7 ± 6.7	129.6 ± 13.6	110.9 ± 15.8	201 ± 12.4*	153.5 ± 18.3	164.0 ± 4.9
AST (U/L)	153.0 ± 13.6	183.7 ± 8.9	202.4 ± 16.2	170.8 ± 9.2	192.5 ± 6.9	191.7 ± 11.3	168.2 ± 12.7	197.1 ± 9.4
ALP (U/L)	239.0 ± 6.9	246.9 ± 8.0	237.1 ± 14.2	241.4 ± 9.4	255.7 ± 9.7	263.3 ± 11.2	243.3 ± 4.6	233 ± 8.8
T-BIL (mg/dl)	0.23 ± 0.05	0.18 ± 0.08	0.35 ± 0.02	0.18 ± 0.13	0.26 ± 0.07	0.17 ± 0.08	0.21 ± 0.05	0.19 ± 0.06
Creatinine (mg/dl)	0.46 ± 0.13	0.31 ± 0.18	0.37 ± 0.10	0.32 ± 0.24	0.56 ± 0.16	0.34 ± 0.13	0.56 ± 0.12	0.33 ± 0.22
BUN (mg/dl)	23.61 ± 0.6	19.49 ± 1.2	23.26 ± 0.2	23.1 ± 0.2	21.87 ± 0.4	20.1 ± 1.6	24.27 ± 1.4	21.08 ± 1.1

All values are expressed as mean ± S.D. * $P < 0.05$ as compared to control

and dose dependent manner. The ROS-mediated induction of oxidative damage and alteration of MMP in the cells may lead to cellular damage, nuclear fragmentation, apoptosis and cell death [23, 24]. During the early phase of apoptosis, the membrane phospholipid phosphatidylserine switches from the inner to the outer leaflet of the plasma membrane [25]. DEGEE mediated apoptosis was confirmed by FITC tagged Annexin V and propidium iodide staining in HEK 293 cells. Thus, the results of this study indicate that DEGEE elucidates its cytotoxicity via the generation of ROS and alteration in MMP which could be a major cause of apoptosis and cell death.

As reported earlier [9], the results of the present study also confirm that DEGEE is non mutagenic at the tested doses since the number of revertant colonies of *Salmonella typhimurium* obtained was not significantly high in both normal and metabolic activation conditions.

In vivo studies were required to monitor and observe the long-term positive and/or adverse effects as the in vitro conditions limit the dispersion, permeability and organ interaction of the test substance [26]. Therefore, *Swiss albino* murine model was selected to conduct further toxicological assessment upon acute and subchronic exposure of DEGEE.

Earlier studies have reported the LD₅₀ of DEGEE in mice via intraperitoneal route to be 3900 mg/kg and 2300 mg/kg [7]. Similar results were observed in the present study, where the survival rates decreased sharply from 3000 to 5000 mg/kg. However, all animals administered with 1000 mg/kg via i.p route survived and showed no behavioural and histological changes when compared to the controls. Therefore, this dose was selected to perform further safety evaluation in a sub-chronic oral repeat dose exposure study.

In the subchronic study, the changes observed in the food and water consumption were not of toxicological relevance

Fig. 5 Effect of oral administration of DEGEE on the food & water consumption and the mean organ weights of *Swiss albino* mice. Statistical analysis of DEGEE treated (a–c) male mice and (d–f) female

mice as compared to control expressed as mean $\pm$ SD ($n = 3$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, calculated compared to control

and can be considered non-adverse since there was no evidence of adverse effects in the hematological and biochemical parameters. Further, an increase in the mean weight of the liver was observed in the male mice receiving 1000 mg/kg DEGEE. These findings are in accordance with the previous study undertaken to evaluate the reproductive effects of DEGEE in CD-1 mice, where the F1 generation mice showed increased relative liver weights [27]. The only relevant histological changes observed were, the increase in

the minimal focal MNCs in kidneys of DEGEE treated mice and minimal dilation of the Bowman's capsule in the females treated with 2000 mg/kg DEGEE. It can be inferred from the results of the subchronic study that the tested doses of DEGEE administered orally showed no signs of adverse toxicity in the murine model. The reason behind this could be the varying extent to which this test substance is absorbed, distributed, metabolized and excreted by an in vivo system.

Table 4 Effect of oral administration of 500–2000 mg/kg DEGEE for 28 days on the organ weight of mice

Dose (mg/kg body weight)	Liver		Kidney		Heart		Brain		Spleen		Lungs	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Control	1.97 ± 0.31	1.72 ± 0.12	0.65 ± 0.03	0.42 ± 0.04	0.19 ± 0.01	0.16 ± 0.02	0.37 ± 0.01	0.37 ± 0.04	0.22 ± 0.02	0.27 ± 0.13	0.32 ± 0.02	0.35 ± 0.06
500 mg/kg	2.13 ± 0.45	1.72 ± 0.05	0.62 ± 0.09	0.45 ± 0.05	0.22 ± 0.03	0.15 ± 0.01	0.42 ± 0.03	0.44 ± 0.02	0.24 ± 0.01	0.29 ± 0.03	0.31 ± 0.16	0.35 ± 0.09
1000 mg/kg	2.32 ± 0.15	1.67 ± 0.23	0.67 ± 0.03	0.40 ± 0.08	0.22 ± 0.04	0.15 ± 0.02	0.34 ± 0.02	0.41 ± 0.05	0.22 ± 0.04	0.23 ± 0.07	0.28 ± 0.02	0.30 ± 0.07
2000 mg/kg	2.10 ± 0.11	1.51 ± 0.14	0.55 ± 0.05	0.39 ± 0.03	0.19 ± 0.02	0.14 ± 0.02	0.43 ± 0.01	0.40 ± 0.05	0.23 ± 0.06	0.22 ± 0.06	0.29 ± 0.09	0.32 ± 0.06

All weights are expressed as mean organ weight in grams ± S.D.

Fig. 6 Effect of oral administration of DEGEE on the histology of the liver, kidney, heart, brain, spleen and lungs of mice treated with 500, 1000, or 2000 mg/kg DEGEE or the vehicle for 28 days. Yellow arrow in the liver indicate areas of minimal hydropic degeneration in hepatocytes. In the kidney, black arrows indicate areas of minimal focal and minimal multifocal MNCs and yellow arrow indicates dilated Bowman's capsule. Images were acquired at 200x magnification; Scale bar = 100 μ m

Conclusion

In summary, a comprehensive evaluation of toxicological parameters of DEGEE indicate that its effects vary depending on the route of administration and duration of exposure. No evidence of nephrotoxicity was observed in the kidneys of subchronically treated mice, except minimal increase of focal MNCs and minimal dilation of Bowman's capsule. However, the decrease in survival rates after acute i.p exposure and the cytotoxic effects of DEGEE in the human embryonic kidney cells indicate that the nephrotoxic potential of this excipient cannot be ruled out. It is clear that additional work and full-scale investigations focussed on DEGEE's toxicological potential and mechanisms of action are required before its use in clinical formulations.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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