Diethylstilbestrol induces oxidative DNA damage, resulting in apoptosis of spermatogonial stem cells *in vitro*

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Highlights

- Exposure of the spermatogonial stem cells to DES produced significant increases in superoxide anion, DNA damage and apoptosis.
- The male reproductive system can be disrupted by foetal exposure to DES.
- The flavonoid quercetin reduced intracellular superoxide anions induced by DES.

Abstract

The spermatogonial stem cells (SSCs) are the only germline stem cells in adults that are responsible for the transmission of genetic information from mammals to the next generation. SSCs play a very important role in the maintenance of progression of spermatogenesis and help provide an understanding of the reproductive biology of future gametes and a strategy for diagnosis and treatment of infertility and male reproductive toxicity. Androgens/oestrogens are very important for the suitable maintenance of male germ cells. There is also evidence confirming the damaging effects of oestrogen-like compounds on male reproductive health. We investigated the effects in vitro, of diethylstilbestrol (DES) on mouse spermatogonial stem cells separated using Staput unit-gravity velocity sedimentation, evaluating any DNA damage using the Comet assay and apoptotic cells in the TUNEL assay. Immunocytochemistry assays showed that the purity of isolated mouse spermatogonial cells was 90%, and the viability of these isolated cells was over 96%. Intracellular superoxide anion production ($O2^{-}$) in SSCs was detected using p-Nitro Blue Tetrazolium (NBT) assay. The viability of cells after DES treatment was examined in the CCK8 (cell counting kit-8) cytotoxicity assay. The results showed that DES-induced DNA damage causes an increase in intracellular superoxide anions which are reduced by the flavonoid, quercetin. Investigating the molecular mechanisms and biology of SSCs provides a better understanding of spermatogonial stem cell regulation in the testis.

1. Introduction

Several compounds in the environment particularly those with estrogenic activity, are able to disrupt the programming of endocrine signalling pathways recognized through development; these compounds are referred to as endocrine-disrupting compounds (Schug *et al.*, 2011). Changed programming can result in numerous adverse consequences in estrogen-target tissues, some of which may not be apparent until later in life (Newbold, 2011). An *in vitro* study by Anderson et al. (Anderson *et al.*, 2003) showed that oestrogens produce reactive oxygen species (ROS), including hydrogen peroxide, at levels that disrupt DNA structure significantly in human sperm and lymphocytes. DES is a synthetic non-steroidal oestrogen that has been used as an extremely effective agent for androgen deprivation therapy in patients with prostate cancer (Kalach *et al.*, 2005; Koong and Watson, 2014), particularly in those who do not respond to other treatments, or those who are castrate-resistant (Grenader *et al.*, 2014; Youn *et al.*, 2012).

DES can bind to estrogen receptors (ERs) in the pituitary and has been widely used as a model estrogen to study alterations in male reproductive function in response to estrogens (Goyal *et al.*, 2001). It also has been shown that DES-induced many abnormalities in the male reproductive system are associated with changed DNA methyltransferase expression and DNA methylation (Sato *et al.*, 2006). DES can mimic estrogen action by interfering with the functioning of the pituitary-gonadal axis, leading to the suppression of testosterone levels that result in increased spermatogenic cell apoptosis (Akingbemi and Hardy, 2001). DES induced germ cell death is a suitable model to study the pathways involved in estrogen induced germ cell apoptosis (Nair and Shaha, 2003). An environmental study has confirmed that exposure to DES caused cancer and functional alterations in the reproductive

endocrine system in male and female mice treated *in utero* (McLachlan, 2016). It also showed that DES stimulation of cell proliferation and gene expression during the binding of the estrogen receptor has been implicated as the inducer of abnormalities caused by estrogens (McKinnell *et al.*, 2001). It has been found that acute exposure to DES induces apoptosis in the testis, and antioxidants play a role in preventing DES-induced testicular tissue damage (Kondo *et al.*, 2002). Studies from our laboratory have shown that quercetin acts as an antioxidant (Cemeli *et al.*, 2004; Dobrzynska *et al.*, 2004) and is an effective free radical scavenger; however, this is the first study where the cytoprotective effect of quercetin on DES-induced toxicity has been studied in isolated spermatogonial stem cells in mouse.

2. Materials and Methods

2.1. Animals

Male adult National Medical Research Institute (NMRI) mice (aged=6-8 weeks old; n=25), derived from the original stocks obtained from the Institute of Cancer Therapeutics Laboratories, were maintained under the standard conditions with free access to food and water at the Animal Facility of the University of Bradford, UK. All animal care procedures were carried out according to the National Research Council's Guide for the Care and Use of Laboratory Animals.

2.2. Isolation and culture of mouse spermatogonial cells

The method for isolation of spermatogonial cells was described previously for the mouse (Habas *et al.*, 2014). Briefly, six testes were collected from three adult NMRI mice (10-12 weeks old), decapsulated, and the seminiferous tubules placed into ice cold Dulbecco's Modified Eagle's medium (DMEM), dispersed by gentle pipetting,

minced and resuspended in fresh DMEM containing collagenase (5mg/ml) and DNase (1µg/ml) (both from Sigma, Poole, UK), then incubated at 32°C for 20 min. A second digestion step was performed in DMEM media by adding fresh enzyme solution into the seminiferous fragments. The cells were left to stand for 5 min before being filtered through an 80µm nylon mesh (Tetco Inc., Briarcliff Manor, NY), centrifuged at 600 × *g* for 10 min and bottom-loaded into the separation chamber of a Staput apparatus in a volume of 10 ml. A 2-4% w/v concentration gradient of Bovine serum albumin (BSA) was then generated below the cells, which were allowed to sediment for a standard period of 2.5 hrs. The cells in each fraction were examined under a phase contrast microscope, and consecutive fractions containing cells of similar size and morphology spun down by low-speed centrifugation and resuspended in DMEM.

2.3. Identification and morphological characteristics of mouse spermatogonial cell

The identity and purity of all cell preparations used in the experiments were confirmed by the immunohistochemistry assay for phase-specific markers exactly as described previously (Habas et al ., 2014). The purity of each population was also confirmed by western blot for the presence or absence of spermatogonia, spermatocyte and spermatid-specific proteins across the 3 independent experiments that were performed as described previously (Habas *et al.*, 2016).

2.4. Determination of cytotoxicity by cell counting kit-8 (CCK-8) assay

Cell viability was determined using a modified cell counting kit-8 (CCK-8) Cytotoxicity Assay (Sigma-Aldrich, UK). Spermatogonial cells were plated in a 96-well plate at a concentration of 5000 cells per well. 10 µl of different concentrations of DES (0, 1, 5 and 10 µM) was added into the culture media in the plate. Cells were pre-incubated

for 24 hrs in a humidified incubator at 37 °C, 5% CO_2. 10 µl of CCK-8 solution was added to each well of the plate, followed by incubation at 37 °C for 4 h. Absorbance was measured at a wavelength of 450 nm using a Microplate reader MRX II (Dynex Technologies, Chantilly, USA).

2.5. The Comet assay

The DNA damage was evaluated by the alkaline version of the Comet assay (pH>13) in spermatogonial cells after treatment with different concentrations of DES and quercetin. Approximately 2 x 10^5 cells per well were incubated with DES at final concentrations of 0, 1, 5 and 10 µM with or without 40 µM quercetin for 1 hr in eppendorf® tubes (5% CO_2, 95%, 37°C). Cell debris was removed by centrifugation and remaining cells were resuspended in 0.5% low melting agarose (LMP Agarose). The pellets from each treatment were harvested by centrifugation and then used for the evaluation of DNA damage using the Comet assay as described previously (Habas et at ., 2016). Olive tail moment (OTM) and % tail DNA were measured, calculated, quantified and expressed (fold of control) in mean ± SEM (n=3) for isolated spermatogonial cells using the (Comet 6.0; Andor Technology, formerly Kinetic Imaging) software image analysis system, Belfast, UK.

2.6. TUNEL staining and quantitation

The apoptotic cells were evaluated on isolated spermatogonial cells by the TUNEL assay using a commercial apoptosis detection kit (Terminal Deoxynucleotidyl Transferase Detection Kit; Promega, UK, Ltd). In brief, approximately 2 x 10^5 cells per well were incubated with DES at final concentrations of 0, 1, 5 and 10 µM with or without 40 µM quercetin at 37 °C for 1 h. The coverslips were incubated with TUNEL reaction mixture (30 mM Tris pH 7.4; 140 mM sodium cacodylate; 1 mM cobalt

chloride; 5 μM biotin-16-deoxyuridine triphosphate; 0.3 U/μl terminal deoxynucleotidyl transferase [TdT]; all from Sigma) for 60 min (humidity chamber, 37 °C) and then washed twice in PBS. Cells were blocked with 0.03% H_2O_2. Cells were washed three times with PBS followed by treatment with 2% v/v Extravidin peroxidase in TBS with 0.1% w/v BSA for 30 min (humidity chamber, 37 °C). Cells were then washed again with PBS, and visualised by adding 3, 3-diaminobenzine (DAB) for 10 min at room temperature. Cells were washed in phosphate buffered saline (PBS); each section counterstained with haematoxylin, then examined and photographed under an Olympus CKX31 microscope (Olympus, Southend on Sea, UK). Negative controls were obtained by incubating sections with the reaction mix in the absence of TdT. Marked condensation of chromatin and cytoplasm clearly staining strongly brown or brown/black were considered as apoptotic cells. The TUNEL positive cells were scored in around 10 fields on coverslips to yield a total of at about 100 cells under 400x magnification.

2.7. Detection of intracellular superoxide anion production

Spermatogonial cells were plated onto 24 well cell culture plates in complete DMEM medium at a density of $5x10^4$ cells per well and incubated at 37^0C and 5% CO2. The cells were washed twice with PBS to remove any dead cells from the plate then spermatogonial cells were treated with 0, 1, 5 and 10μM DES for 1 hr. Medium then was aspirated and replaced with phenol red-free media (M199) (Sigma-Aldrich, UK), containing 1 mg/ml NBT with and without 1, 5 and 10μM DES. The cells then were incubated at 37^0C and 5% CO_2 for 90 min. Spermatogonial cells were directly lysed with lysis solution (90% DMSO, 0.1% SDS and 0.01 M of NaOH). The resulting blue coloured solution was measured spectrophotometrically at a wavelength of 750nm using microplate reader MRX II (Dynex Technologies, Chantilly, USA). The

production of intracellular superoxide anion was proportional to the absorbance values at 750nm.

2.8. Statistical analysis

Data are expressed as mean ± SEM of at least three independent experiments with three replicates per experimental group. Comparisons were made by one-way ANOVA followed by Bonferroni post-hoc test; for all experiments, a *p* value of <0.05 was considered significant.

3. Results

3.1. Cytotoxicity Assay

The cytotoxicity assay using CCK-8 kit was performed to directly determine the effect of DES on cellular viability of spermatogonia under our laboratory conditions. Spermatogonia were either treated with different concentrations of DES (0, 1, 5 and 10μM) or left untreated and considered as control. The result showed that DES at the concentrations 0, 1 and 5 μM had no significant effect on cell viability. In contrast, at 10 μM there was a significant effect on cell viability of spermatogonial cells (*$p \leq 0.05$) at 24hrs (Fig.1).

3.2. Detection of DNA damage by the Comet assay

Olive tail moment (OTM) and %tail DNA are shown in Figs. 2 and 3 respectively. A significant increase from 0.92 in control to 3.73 (OTM) and 7.23 in control to 21.29 (% tail DNA) was observed in the OTM and % tail DNA of spermatogonia treated with 5 μM DES. At 10 μM, OTM and % tail DNA damage also showed a significant increase to 6.34 and 26.10% respectively in spermatogonial cells compared with control (**$p \leq 0.01$ and ***$p \leq 0.001$ respectively). Spermatogonial cell treated with 5

µM DES and 40 µM quercetin showed significant decreases in OTM and % tail DNA from 3.73 to 1.85 (OTM) and 21.29 to 11.53 (% tail DNA) respectively ($^{\#}p \leq 0.05$). At 10 µM, OTM and %tail DNA damage also showed a significant decreases from 6.34 to 2.09 and 26.10 to 13.00% respectively in spermatogonial cells compared with treated cells with 10 µM of DES alone ($^{\#\#\#}p \leq 0.001$ and $^{\#\#}p \leq 0.01$ respectively).

3.3. Detection of apoptosis by the TUNEL assay

The result of DES treatment on isolated spermatogonial cells of mouse testis was expressed as mean percentage per group ± SEM of apoptotic cells. The TUNEL assay showed that spermatogonial cells had undergone significant levels of apoptosis compared with the controls ($^{*}p \leq 0.05$) (Fig. 4). After treatment with DES (0, 1, 5 and 10 µM) for 1h, a significant increase ($^{*}p \leq 0.05$) in spermatogonial apoptosis from 6.33 in control to 10.00 % was observed when cells were treated with 5 µM DES. This increased to 14.33 % when treated with10 µM DES ($^{*}p \leq 0.05$). Spermatogonial cell treated with 5 µM DES and 40 µM quercetin showed that quercetin treatment resulted in a significant reduction in apoptotic cells from 10.00 to 6.67 compared with 5 µM DES alone ($^{\#}p \leq 0.05$). At 10 µM DES and 40 µM quercetin, a significant reductions from 14.33 to 9.00 was observed when compared with 10 µM DES alone ($^{\#\#}p \leq 0.01$).

3.4. Detection of intracellular superoxide anion production

The NBT assay was used specifically to measure superoxide anion generation. Cells treated with DES for 1hr showed clear increased production of superoxide anions which were significantly increased compared to non-treated cells ($^{*}p<0.05$), ($^{**}p<0.01$) and ($^{***}p<0.001$) (Fig. 5). Therefore, co-treatment with quercetin and DES was studied in spermatogonial cells in order to investigate whether generation might

reduce the increases in the generation of superoxide oxidase anion production. Cells treated with 1µM DES and 40µM quercetin showed that quercetin treatment resulted in a significant reduction in O_2^- levels compared with 1µM DES alone ($^{\#\#}p \leq 0.01$). Cells treated with 5 µM DES and 40µM quercetin also showed a significant reduction in O_2^- levels compared with 5 µM DES alone ($^{\#}p \leq 0.05$). Further reduction in O_2^- levels was also shown when cells were treated with 10 µM DES alone ($^{\#\#}p \leq 0.01$), all results shown in Fig. 5.

4. Discussion

The aim of the present study was to evaluate the DNA damage induced by DES and apoptotic cells in the TUNEL assay, as well as intracellular superoxide anions using the NBT assay in isolated spermatogonial cells. This study was also performed to investigate effects of quercetin on oxidative damage that was induced by DES in cultured spermatogonial cells of mouse testis. A number of previous studies have shown that the toxic effects of environmental oestrogens on male reproductive health are commonly performed *in vivo*. There are limited toxicity data *in vitro* (Anderson, Schmid, Baumgartner, Cemeli-Carratala, Brinkworth and Wood, 2003; Li *et al.*, 2010). It has been shown that estrogens induce apoptosis in male germ cells, but the pathways leading to apoptotic death from exposure to estrogens has not been fully understood (Nair and Shaha, 2003). A number of studies showed that cellular apoptosis in the testis in different model systems has used total testicular tissue and not isolated germ cells to separate pathways leading to cell death (Eid *et al.*, 2002; Yin *et al.*, 2002).

Using isolated male germ cells may provide a completely different understanding, as the total testicular tissue is a heterogeneous organ composed of multiple cell types

(Nair and Shaha, 2003). In this present study present, spermatogonial cells were isolated and cultured *in vitro*. Our results showed that DES produced DNA strand breaks in a concentration dependent manner in spermatogonial cells. This induction of DNA strand breaks is significant in that it shows how DES affects normal cells which could cause genetic damage that may be lead to cancer. A previous study has shown that DES undergoes redox cycling producing DES quinone and oxygen radicals; resulting in the production of adducts (Anderson, Schmid, Baumgartner, Cemeli-Carratala, Brinkworth and Wood, 2003; Thomas *et al.*, 2004). These results are supported by our present data which showed that exposure to DES had statistically significant increased DNA damage which could be the result of the production of superoxide by DES redox cycling. Studies have shown that exposure to DES might generate ROS (Anderson, Schmid, Baumgartner, Cemeli-Carratala, Brinkworth and Wood, 2003) and increased lipid peroxidation in rat and hamster models (Ma *et al.*, 2008).

The NBT results showed that high levels of O_2^- were formed in spermatogonial cells treated with 10 µM DES. DNA damage can be attenuated by antioxidants. This study focused on quercetin as an antioxidant. Quercetin is an important flavonoid that has multiple beneficial biological, pharmacological, and medicinal properties including anti-inflammatory and cytoprotective effects (Gonzalez-Gallego *et al.*, 2010). It has been shown to reduce oxidative stress-dependent damage in both *in vitro* (Anderson, Schmid, Baumgartner, Cemeli-Carratala, Brinkworth and Wood, 2003; Liu *et al.*, 2010), and *in vivo* (Barcelos *et al.*, 2011). O_2^- levels were reduced when the spermatogonial cells were simultaneously exposed to DES. O_2^- was significantly reduced in the concomitant treatment of cells with 1, 5 and 10 µM DES and 40 µM quercetin ($^{\#\#}p<0.01$).

These results are interesting for various important reasons. Our study using the in vitro model of spermatogonial stem cells could make it easier to understand the mechanisms underlying spermatogonia cell differentiation. Extrapolating to humans will be very useful, in which experimental approaches are limited. Furthermore isolated male germ cells could greatly improve and make the actual procedures of assisted reproductive technology more efficient and develop alternative infertility treatments. This technique is not new but it is rapid and does not need excessive animal experimentation *in vivo*, in contrast to traditional techniques that employ histological morphology and fertility studies. Furthermore, since this study only involves short term culture, it enables detection of the earliest signs of toxicity. This makes it useful for germline genotoxicity for drugs and other compounds of interest, and hence of value in safety evaluation procedures.

Conclusions

The results presented in this study showed that DES can adversely damage the spermatogonial stem cells by increasing DNA damage, apoptosis and superoxide anions. They also showed that quercetin had a protective effect on spermatogonial cells against oxidative damage and apoptosis caused by DES, which is an important flavonoid-reducing ROS and reproductive toxicity can be caused by this environmental oestrogen.

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Conflict of Interest Statement

The corresponding author confirmed that the authors have no conflicts of interest with regard to the funding of this research.

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Figure Legends

Fig. 1 Assessment of cytotoxicity of different concentrations of DES for 24hrs on spermatogonial cells using the cytotoxicity assay (CCK-8).

Fig. 2 Induced DNA damage in spermatogonial cells after treatment with different concentrations of DES (1, 5 and 10 µM) alone or with 40 µM quercetin for 1h. OTM was used for DNA damage quantification. *P <0.05, **P <0.01, *** P <0.001 when compared with the respective control group. $^{\#}p$<0.05, and $^{\#\#\#}p$<0.001 indicates cells treated with 1, 5 and 10 µM DES and 40 µM quercetin for 1hr.

Fig. 3 Induced DNA damage in spermatogonial cells after treatment with different concentrations of DES (1, 5 and 10 µM) alone or with 40 µM quercetin for 1h. %Tail DNA was used for DNA damage quantification. *P <0.05, **P <0.01, *** P <0.001 when compared with the respective control group. $^{\#}p$<0.05, and $^{\#\#}p$<0.01 indicates cells treated with 1, 5 and 10 µM DES and 40 µM quercetin for 1hr.

Fig. 4 Effect of DES treatment on spermatogonial cells assessed using the TUNEL assay. Data shows the mean percentages ± SEM of apoptotic cells at DES concentrations of 1, 5 and 10 µM alone or with 40 µM quercetin for 1h. *P < 0.05, **P < 0.01, *** P < 0.001 when compared with the respective control group. $^{\#}p$<0.05, and $^{\#\#}p$<0.01 indicates cells treated with 1, 5 and 10 µM DES and 40 µM quercetin for 1hr.

Fig. 5 Effect of DES and quercetin co-incubation on superoxide anion production in spermatogonial cells using the NBT assay. Spermatogonial cells were grown in complete medium and treated with and without 1, 5 and 10µM DES alone or DES and 40 µM quercetin for 1hr. Non-treated cells were considered as negative control. The results are expressed as mean (± SEM) from 3 different donors (n=3). *p<0.05,

**p<0.01 and ***p<0.01 versus non-treated cells. $^{\#}p$<0.05, and $^{\#\#}p$<0.01 indicates cells treated with 1, 5 and 10 µM DES and 40 µM quercetin for 1hr.

Fig. 1

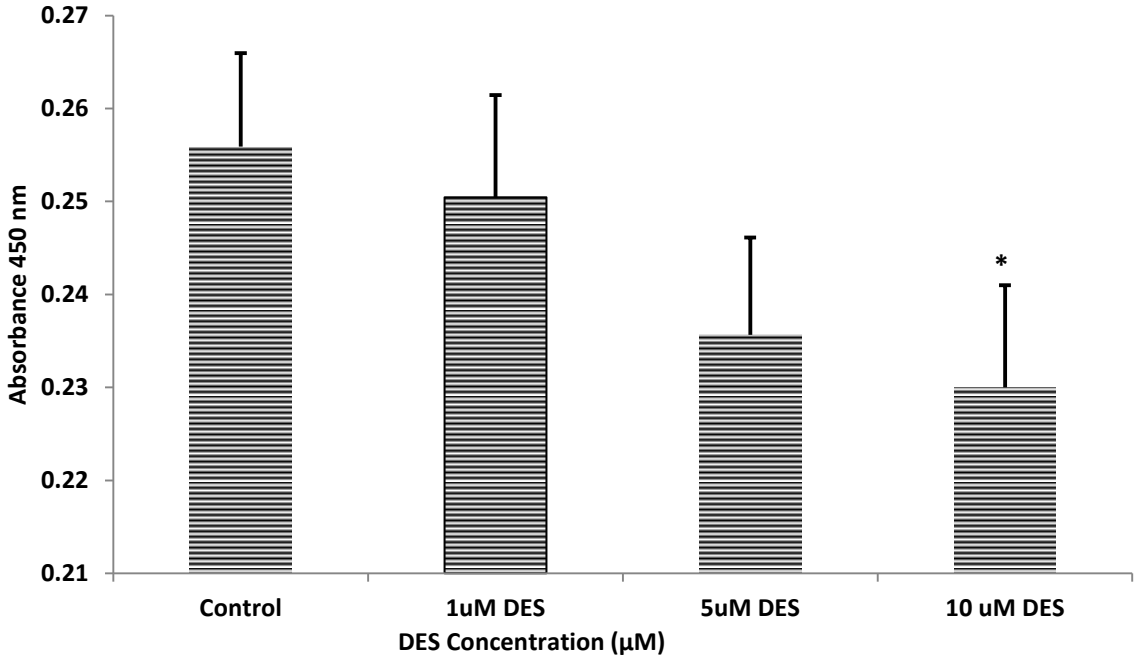

Fig. 2

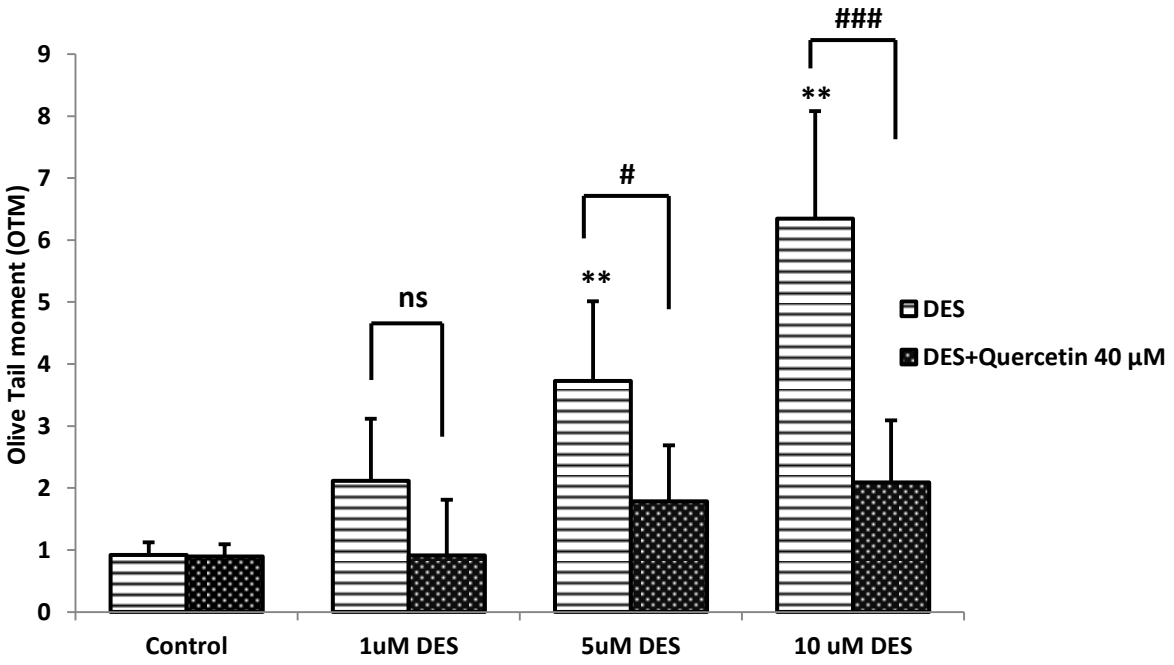
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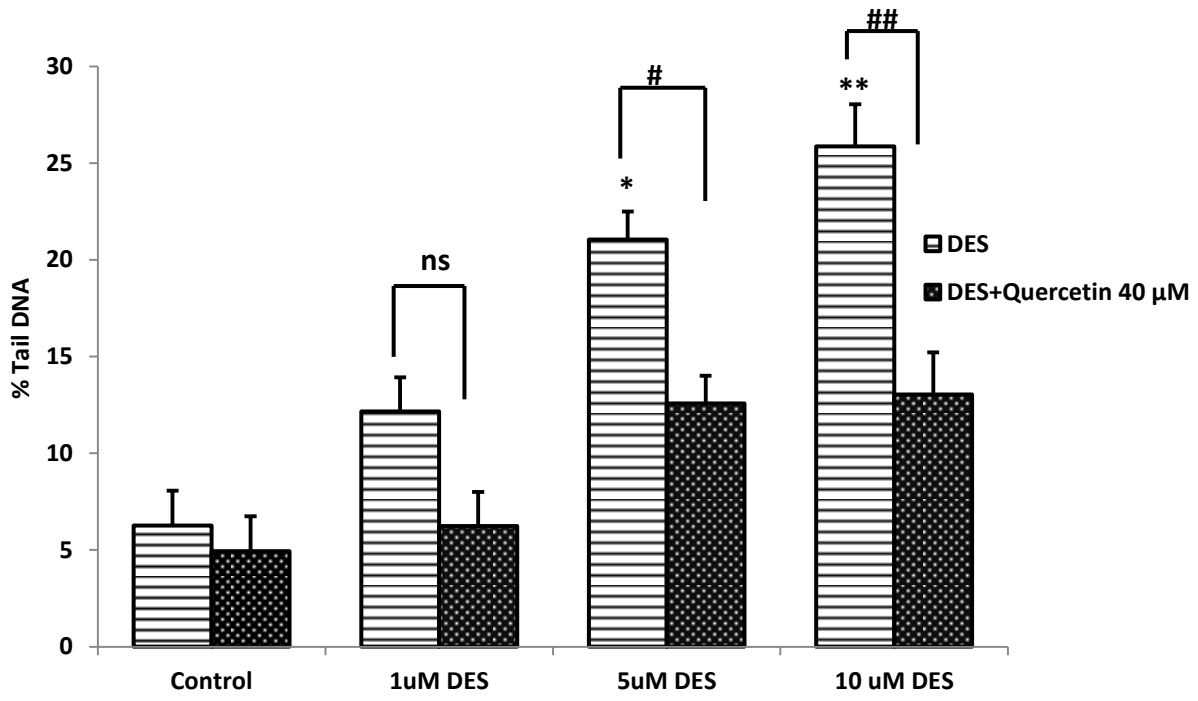
Fig. 3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10 uM DES

Fig. 4

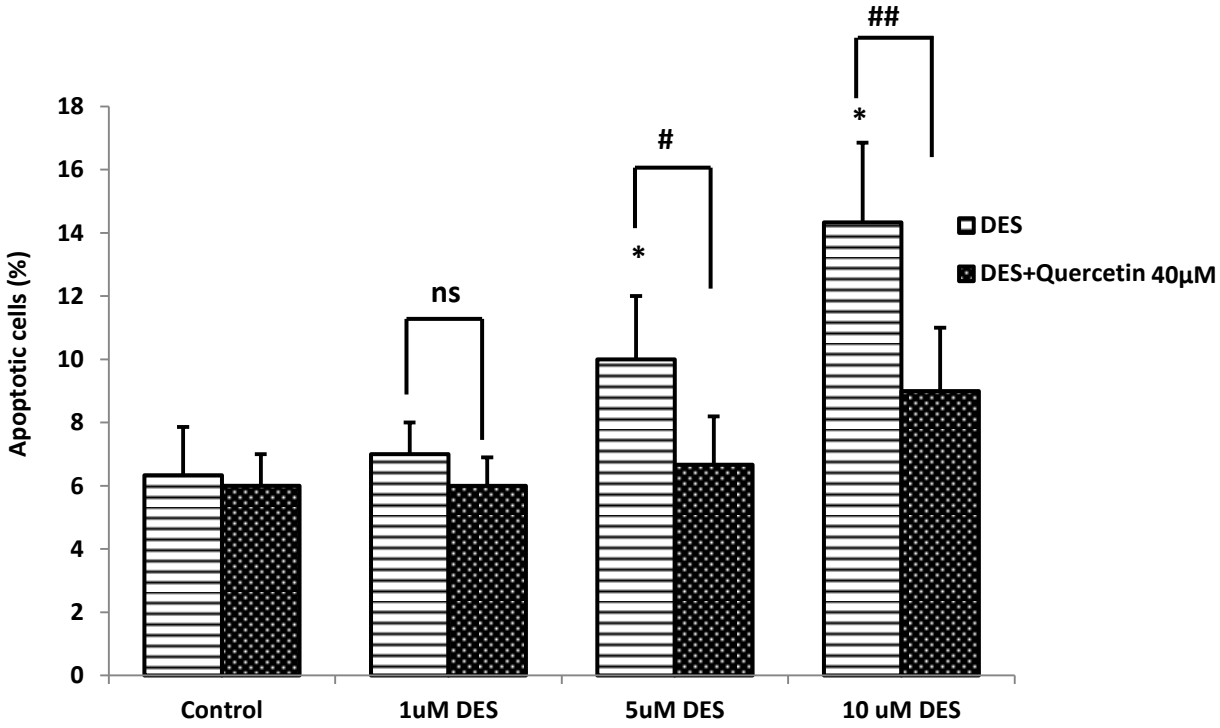
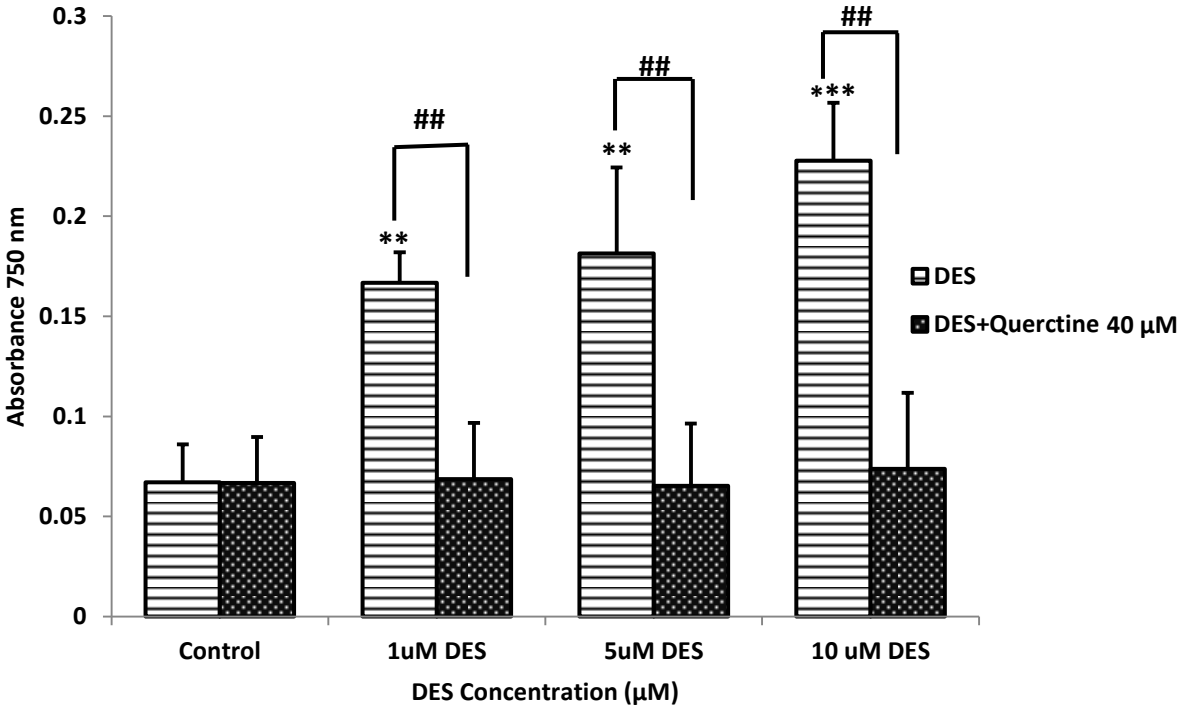

Apoptotic cells (%)
18
16
14
12
10
8
6
4
2
0
ns
*
#
*
##
DES
DES+Quercetin 40µM
Control
1uM DES
5uM DES
10 uM DES

Fig. 5


0.3
0.25
0.2
0.15
0.1
0.05
0
Absorbance 750 nm
**
##
**
##
***
##
Control
1uM DES
5uM DES
10 uM DES
DES Concentration (µM)
DES
DES+Querctine 40 µM