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ORIGINAL RESEARCH

Can dicoumarol be used as a gonad-safe anticancer agent: an *in vitro* and *in vivo* experimental study

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STUDY HYPOTHESIS: Dicoumarol (DC) has potential for use as a gonad-safe anticancer agent.

STUDY FINDING: DC altered cell proliferation, decreased viability and increased apoptosis in Vero and MCF-7 cell lines but did not show any toxic effect on mouse ovarian tissues and developing oocytes *in vitro* and *in vivo*.

WHAT IS KNOWN ALREADY: DC suppresses cell proliferation and increases apoptosis in various cancer cells such as breast, urogenital and melanoma. DC has also been reported to alter the anticancer effects of several chemotherapeutics, including cisplatin, gemcitabine and doxorubicin in prostate, liver and uroepithelial cancer cells, respectively.

STUDY DESIGN, SAMPLES/MATERIALS, METHODS: Vero (African green monkey kidney epithelial cells) and MCF-7 (human cancerous breast epithelial cells) cell lines and mouse granulosa cells isolated from 21-day-old female BALB/c mice ($n = 21$) were used to assess the effects of DC (10, 50, 100 and 200 μM) for 24 and 48 h on cell proliferation, viability and apoptotic cell death. *In vivo* experiments were performed with a single i.p. injection of 32 mg/kg DC in 21-day-old female BALB/c mice ($n = 12$). Following 48 h, animals were sacrificed by cervical dislocation and histological sections of isolated ovaries were evaluated for apoptosis. Viability assays were based on the trypan blue dye exclusion method and an automated cell counter device was used. Terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling (TUNEL) and Annexin-V immunofluorescence were assessed by 3D confocal microscopy to address apoptotic cell death. We also assessed whether DC inhibits cell proliferation and viability through NQO1 [NAD(P)H Quinone Oxidoreductase 1], an intracellular inhibitor of reactive oxygen species (ROS). The meiotic spindle and chromosomes were studied in mouse oocytes by α-β-tubulin and 7-aminoactinomycine D (7-AAD) immunostaining *in vitro* and *in vivo*.

MAIN RESULTS AND THE ROLE OF CHANCE: DC does not block oocyte maturation and no significant alteration was noted in meiotic spindle or chromosome morphology in metaphase-II (M-II) stage oocytes following DC treatment *in vitro* or *in vivo*. In contrast, exposure to DC for 24 h suppressed cell proliferation ($P = 0.026$ at 200 μM), decreased viability ($P = 0.002$ at 200 μM) and increased apoptosis ($P = 0.048$ at 100 μM) in Vero and MCF-7 cell lines, compared with controls. These changes were not related to intracellular NQO1 levels. Mouse granulosa cells were unaffected by 50 or 100 μM DC treatment for 24 and 48 h *in vitro*. DC treatment *in vivo* did not alter the number of primordial follicles or the ratio of apoptosis in primordial, primary and secondary follicles, as well as in antral follicles, compared with the controls.

LIMITATIONS, REASONS FOR CAUTION: DC was tested for ovarian toxicity only in isolated mouse oocytes/ovaries and healthy BALB/c mice. No cancer formation was used as an *in vivo* test model. The possibility that DC may potentiate ovarian toxicity when combined with traditional chemotherapeutic agents, such as mitomycin-C, cisplatin, gemcitabine and doxorubicin, must be taken into account, as DC is known to alter their effects in some cancer cells.

WIDER IMPLICATIONS OF THE FINDINGS: The present study evaluated, for the first time, the effect of DC on ovarian tissue. The results suggested that DC is not toxic to ovarian tissues and developing oocytes; therefore, DC should be assessed further as a potential anticancer agent when female fertility preservation is a concern.

LARGE SCALE DATA: N/A.

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STUDY FUNDING AND COMPETING INTEREST(S): This work includes data from dissertation thesis entitled 'Effects of dicoumarol on mitotic and meiotic cells as an anticancer agent' by DA, 2014 and was partly supported by The National Scientific and Technological Research Council of Turkey (SBAG-109S415) to AC, OC and SO. The authors confirm that this article content presents no conflicts of interest.

Key words: apoptosis / dicoumarol / fertility preservation / meiosis / oocyte

Introduction

A coumarin derivative, dicoumarol (DC), chemically termed as 3,3′-methylenebis (4-hydroxycoumarin) shows cytotoxic effects on several malignant cell types as previously reported in various *in vitro* (Moran *et al.*, 1993; Siegers and Bostelmann, 1993; Marshall *et al.*, 1994; Finn *et al.*, 2001) and *in vivo* (Feuer *et al.*, 1976; Thornes *et al.*, 1989) studies and is considered as an anticancer agent. For example, DC was shown to suppress cell proliferation and induce apoptosis in pancreatic cancer cells (Cullen *et al.*, 2003; Lewis *et al.*, 2004). Similarly, it was shown that DC inhibits cell proliferation in certain human breast (Du *et al.*, 2006), urogenital (Watanabe *et al.*, 2006) or melanoma (Brar *et al.*, 2001) cancer cell lines.

One mode of action of DC is to inhibit the NQO1 [NAD(P)H Quinone Oxidoreductase 1] protein (Scott *et al.*, 2011), an intracellular quinone oxidoreductase which reduces ROS production (Begleiter and Fourie, 2004; Lewis *et al.*, 2005), as well as p53 stabilization (Asher *et al.*, 2001, 2002; Anwar *et al.*, 2003). DC has also recently been suggested as a microtubule stabilizing agent because it blocks the first division of sea urchin embryos in a dose-dependent manner (Jacobs *et al.*, 2003; Madari *et al.*, 2003). Independent studies suggest that the anticancer effect of DC is executed through the inhibition of NQO1 (Cullen *et al.*, 2003; Du *et al.*, 2006) or the stabilization of microtubules (Jacobs *et al.*, 2003); however, the mechanisms of action remain to be determined. Microtubule stabilizing anticancer agents, such as paclitaxel, induce apoptosis through a distinct pathway (Wang *et al.*, 1999) and cause gonadal failure by primordial follicle loss (Turkyilmaz *et al.*, 2008). No report has been published to date that examines the effects of DC on ovaries and oocyte maturation. The aim of the present study was to address the potential toxicity of DC administration on mouse ovaries and oocytes when used in previously reported anti-proliferative doses. For this purpose, meiotic cell division, cell proliferation and apoptosis in DC-treated mouse oocytes and granulosa cells were assessed *in vitro* and *in vivo*. The anti-proliferative and apoptotic effects of DC were also tested on Vero and MCF-7 cell lines, which represent normal and cancerous epithelial somatic cells. The mode of action on DC cytotoxicity was also investigated through comparative analysis of the mitotic cell index and NQO1 expression.

Materials and Methods

Isolation of mouse cumulus–oocyte complexes and somatic cell cultures

Ethical approval was obtained from the Local Ethical Board of Animal Experiments (approval no. 2013-14-98). All reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise stated. Vero and MCF-7 cell lines were obtained from The Institute of Foot and Mouth Diseases (registration no. 97121501 and 00092502, respectively). Ovarian follicular development was stimulated by the i.p. injection of pregnant mare's serum gonadotrophin (0.5 IU/100 μL) in 21-day-old female BALB/c mice ($n = 33$). After sacrificing the animals by cervical dislocation, oocytes and surrounding cumulus cells (cumulus–oocyte complexes, COCs) were collected from the isolated ovaries by follicular puncture. Germinal Vesicle (GV)-stage oocytes were transferred into the gamete incubation medium G-IVF Plus (Vitrolife, Sweden) for maturation, following denudation with gentle pipetting.

Vero, MCF-7 cell lines and isolated granulosa cells from mouse ovaries were cultured in a 1:1 mixture of Dulbecco's Modified Eagle's Medium/Nutrient Mixture Ham's F-12 (DMEM/F12) supplemented with 10% heat-inactivated foetal bovine serum, 1% (w/v) penicillin–streptomycin and 2.5 mg/ml amphotericin B in a humidified atmosphere of 5% CO_2 at 37°C.

DC experiments

DC (purity ≥ 98%, MW 336.29 g/mol) (Sigma–Aldrich, St. Louis, MO, USA) was dissolved in ultrapure water (pH: 11.9) to prepare a 10 mM stock solution. Working solutions were freshly prepared and dissolved in either G-IVF Plus or DMEM/F12 media.

Two different sets of *in vitro* and *in vivo* experiments were carried out to assess the effects of DC on oocyte maturation. GV-stage oocytes ($n = 120$) were either immersed in maturation medium containing 100 μM DC for 18 h or microinjected with 100 μM DC using an inverted microscope (Olympus, Melville, NY, USA) equipped with a micromanipulation system (Eppendorf, Germany) and a heating stage (Olympus, Melville, NY, USA). Control group COCs ($n = 60$) were also microinjected or immersed in maturation medium lacking DC. Mature oocytes were fixed with a microtubule stabilizing buffer that contained 2% formaldehyde and 0.1% Triton-X for 30 min at 37°C (Cinar *et al.*, 2015). *In vivo* experiments were performed with a single i.p. injection of 32 mg/kg DC to 21-day-old female BALB/c mice. Ovaries were removed 48 h after the injection and were either fixed in 10% formalin for TUNEL assay or used for the isolation of COCs. Maturation of GV-stage oocytes was observed for the following 18 h, and at the end of the maturation period, they were fixed for fluorescent labelling as described later.

Three sets of experiments were carried out with Vero, MCF-7 and primary cultures of granulosa cells using increasing doses of DC. Cells were cultured on glass coverslips in 24-well culture dishes with an initial number of 0.02×10^6 cells/well. Following 30 h of plating, three replicates of each cell type were treated with 0 (control), 10, 50, 100 or 200 μM DC and monitored for the following 24 or 48 h. One group of cells was detached from the culture plate with trypsin-EDTA solution (Biochrom, Germany), (5 min at 37°C) to perform proliferation and viability assays. Another group of cells was fixed in 3.5% paraformaldehyde (PFA) for 20 min at room temperature, for further analysis (see below). As a positive control, granulosa cells were treated with 20 μg/ml busulphan, a well-known alkylating chemotherapeutic agent, for 8 h to mimic the microtubule-independent inhibition of cell proliferation (Hassan *et al.*, 2001) (data not shown).

Vero and MCF-7 cells with an initial number of 0.3×10^6 cells/flask were cultured in 75 cm^2 flasks. Cells were exposed to no DC (control), or 50 or 100 μM DC after 30 h of culture for 24 h, washed twice in Dulbecco's Phosphate-Buffered Saline (PBS) and stored at −80°C until processing for western blot analysis.

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Labelling of the oocytes and image acquisition

Fixed oocytes were incubated in a 1:1 mixture of anti-α-tubulin/anti-β-tubulin mouse monoclonal antibodies (1:100 dilution), for 90 min at 37°C, for the assessment of meiotic spindle integrity. Oocytes were then washed in PBS and incubated in a 1:100 dilution of an affinity-purified fluorescein isothiocyanate (FITC) goat anti-mouse immunoglobulin (Ig) G (Jackson ImmunoResearch, PA, USA) for 60 min at 37°C. For the evaluation of chromosome distribution, oocytes were dual-stained with 10 μM 7-AAD or 1 μg/ml Hoechst 33258 (Molecular Probes, Eugene, OR, USA) as nuclear stains. Finally, oocytes were mounted between glass slides and coverslips with a 1:1 mixture of glycerol/PBS solution containing 25 mg/ml sodium azide as an anti-fading reagent.

Slides were examined with a Carl-Zeiss (Germany) laser scanning confocal microscope (LSM-510) equipped with 488-nm Argon ion, 543-nm He-Ne and 633-nm He-Ne lasers and a 63× Zeiss Plan-Apo objective. Single and z-axis optical sections for 3-D image constructions were collected and images were recorded using LSM-510 software (Carl Zeiss, Germany).

Determination of cell proliferation, viability and mitotic cell index

Viability of trypsinized Vero, MCF-7 and granulosa cells was assessed with an automated cell counter device (Vi-Cell, Beckman Coulter, USA) based on the trypan blue dye exclusion assay. The Vero and MCF-7 cells, which had been fixed on glass coverslips, were then labelled with a monoclonal mouse antibody against Ki-67 antigen (1:100 dilution), a nuclear marker for proliferation. Cells were then washed in PBS and incubated in 1:100 dilution of an FITC conjugated goat anti-mouse (Ig)G, for 60 min at 37°C. Chromosomes were labelled with 7-AAD.

For the determination of mitotic cells, Vero, MCF-7 and granulosa cells were fixed on glass coverslips and labelled with 1 μg/ml Hoechst 33258. Approximately 1000 cells were counted on each coverslip and mitotic figures from prophase to telophase were recorded. The fraction of dividing cells relative to the total number of cells was taken as the mitotic cell index.

Measurement of apoptosis

For the demonstration of DNA strand breaks in apoptotic cells, the TUNEL assay was performed with a fluorescein *in situ* cell death detection kit (Roche, Germany). A 1:10 mixture of a TdT enzyme solution and fluorescein-conjugated nucleotides was prepared. Vero, MCF-7 and granulosa cells fixed on glass coverslips or PFA-fixed 4 μm-thick cryosections of ovaries were incubated in this mixture, for 60 min at 37°C. The glass coverslips and slides were then washed in PBS and chromosomes were marked with 7-AAD, to ensure the nuclear localization of DNA strand breaks. Approximately 1000 cells were counted on each coverslip. The fraction of TUNEL-labelled ($TUNEL^+$) cells relative to the total number of cells was taken as the apoptotic cell index (TUNEL index). Similarly, TUNEL index was calculated in serial sections for each individual follicle (primordial, primary, secondary and antral), as well as in corpus luteum.

For the validation of TUNEL data, externalization of phosphatidylserine molecules to the outer plasma membrane of apoptotic cells was detected with Annexin-V labelling. The glass coverslip-fixed Vero, MCF-7 and granulosa cells were incubated in 1:100 dilution of an Annexin-V mouse monoclonal antibody, for 60 min at 37°C. Fluorescein-conjugated secondary antibody (goat anti-mouse) and nuclear labelling were applied as described earlier. Approximately 1000 cells were counted on each coverslip. The fraction of Annexin-V labelled ($Annexin\text{-}V^+$) cells relative to the total number of cells was calculated.

Detection and quantification of NQO1 protein

The coverslip-bound Vero, MCF-7 and granulosa cells were incubated in a 1:100 dilution of a NQO1 mouse monoclonal antibody for 60 min at 37°C. Cells were then washed in PBS, incubated in a goat anti-mouse fluorescein-conjugated secondary antibody and chromosomes were labelled, as described earlier. To quantify the NQO1 positivity in labelled granulosa cells, varying signal intensities were analysed using ImageJ v.3.91 software (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). The formula used to calculate the corrected total cell fluorescence (CTCF) was CTCF = integrated density − (area of selected cell × mean fluorescence of background readings) (Burgess *et al.*, 2010; Cinar *et al.*, 2015; Coskun and Can, 2015).

Western blotting was used for the semi-quantification of NQO1 protein in Vero and MCF-7 cells. Total cell extracts were obtained from cultured cells using a cell lysis buffer (Invitrogen, Carlsbad, CA, USA) containing 2% protease inhibitor cocktail. The total protein concentration was determined using BCA (Bicinchoninic Acid) protein assay. Proteins (50 μg/lane) were separated with 10% SDS–polyacrylamide gel electrophoresis and electroblotted to PVDF (Polyvinylidene Fluoride) membranes (Bio-Rad, Hercules, CA, USA). Membranes were washed in distilled water and incubated in a blocking solution of 5% (w/v) non-fat dry milk in Tris-Buffered Saline (TBS) containing 0.1% Tween 20 (TBS-T) (Bio-Rad, Hercules, CA, USA) for 60 min at room temperature. Membranes were then incubated in an NQO1 mouse monoclonal antibody (1:1000 dilution) overnight at 4°C and washed twice in TBS-T. Blots were then incubated in a 1:3000 dilution of a horse-radish peroxidase goat anti-mouse (Ig)G for 2 h at room temperature, washed twice in TBS-T, and a chemiluminescence reagent (Thermo Fisher Scientific, Waltham, MA, USA) was applied for 5 min at room temperature in a dark room. Antibody-bound bands were visualized on 20 × 20-cm films (Amersham Biosciences, UK), and densitometric measurements of protein bands were analysed using ImageJ v.3.91 software (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Results were shown as mean and standard deviation for three biologic or experimental replicates. Statistical analyses were performed using the computer-based software package (version 15.0; SPSS, Inc., Chicago, Il, USA). Normally distributed data were analysed with one-way ANOVA (Analysis of Variance) and Fisher's LSD (Least Significant Difference) post-hoc tests and non-parametric analysis were performed with Chi-square (χ^2) test. A value of $P < 0.05$ was considered statistically significant.

Results

Effects of DC on cell proliferation

Vero, MCF-7 and granulosa cells were cultured for 96 h to determine their proliferative dynamics. Vero and MCF-7 cells entered the *log* phase in the 30 h of culture, after which DC was applied. The number of granulosa cells in culture remained constant for 96 h. Population doubling times for Vero and MCF-7 cells were calculated as 25 and 22 h, respectively (Fig. 1A).

To address the DC effect on cell proliferation, the comparative IC_{50} of cell proliferation analyses were performed using a downward sloping dose–response curve in DC-treated groups for Vero and MCF-7 cells. IC_{50} represents the concentration of a drug that is required for 50% inhibition *in vitro*. Thus, the IC_{50} was calculated as 50 and 40 μM for Vero and MCF-7 cells, respectively. Based on the above IC_{50} values, the granulosa cells were treated with 50- and 100 μM DC. Untreated Vero cells increased ~2-folds during 24 h of culture, while DC-treated cells decreased in a dose-dependent manner (Fig. 1B). The decrease in cell number was significant between the control and 200 μM DC-treated groups ($P = 0.026$) over the first 24 h. The linear increase of cell

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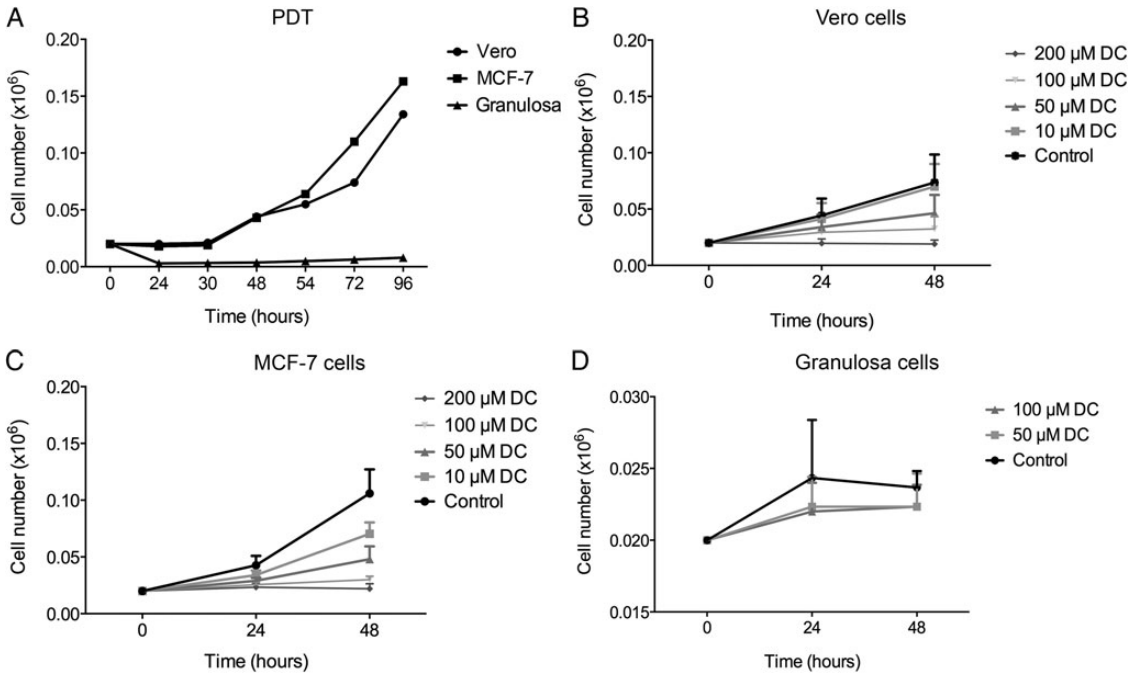

Figure 1 Proliferation of DC (dicoumarol)-treated Vero, MCF-7 and granulosa cells. Proliferation characteristics of Vero, MCF-7 and granulosa cells for 96 h in culture (**A**) were assessed by Population Doubling Time (PDT; the period of time required for the growth of a population to double). Vero and MCF-7 cells spanned the first 30 h in *lag phase* and subsequently entered *log phase* during which a steep increase was noted during the tested time interval. Granulosa cells did not display any significant proliferation rate throughout the 96-h culture period. For the assessment of IC_{50} (the concentration of DC that is required for 50% inhibition *in vitro*), Vero (**B**) and MCF-7 (**C**) cells were treated with increasing doses (10, 50, 100 and 200 µM) of DC. Significant differences were noted in Vero cells (B) as follows: $P = 0.026$ between control versus DC (200 µM) in 24 h; $P = 0.013$ between control versus DC (100 µM) in 48 h; $P = 0.003$ between control versus DC (200 µM) in 48 h. In MCF-7 cells, (C) significant differences were noted as follows: $P = 0.045$, $P = 0.005$, $P = 0.001$ and $P < 0.001$ for 10, 50, 100 and 200 µM doses versus control, respectively in 24 h; $P = 0.004$, $P < 0.001$, $P < 0.001$ and $P < 0.001$ for 10, 50, 100 and 200 µM doses versus control, respectively in 48 h. Vero (B) and MCF-7 (C) cell numbers decreased linearly as the dose and time increased, whereas cell proliferation rate did not change in 50- and 100 µM DC-treated granulosa cells (**D**). Results were shown as mean and standard deviation for three experimental replicates. Normally distributed data were analysed with one-way ANOVA and Fisher's LSD post-hoc tests.

number in the control group continued for 48 h, whereas a linear decrease was noted in DC-treated Vero cells, which showed a significant reduction in cell number at 100 µM ($P = 0.013$) and at 200 µM ($P = 0.003$) compared with the control group (Fig. 1B). MCF-7 cell proliferation significantly decreased with increasing DC doses during the first 24 h ($P = 0.045$, $P = 0.005$, $P = 0.001$ and $P < 0.001$ for 10, 50, 100 and 200 µM doses, respectively) and further decreased during the 48 h of culture ($P = 0.004$, $P < 0.001$, $P < 0.001$ and $P < 0.001$ for 10, 50, 100 and 200 µm doses, respectively) (Fig. 1C, Supplementary data, Video). The number of granulosa cells did not change significantly in both control and DC-treated groups at 24 and 48 h (Fig. 1D). Proliferating cell numbers were determined using Ki-67 staining, which was typical in proliferating cells in the control group, while none of the DC-treated Vero or MCF-7 cells showed Ki-67 positivity (Fig. 2). These findings demonstrated that DC blocks proliferation in Vero and MCF-7 cells in a time- and dose-dependent manner, but does not diminish the number of granulosa cells *in vitro*.

Determination of the mitotic cell index

To examine whether the anti-proliferative action of DC is a mitotic phase-related effect, the mitotic cell index was determined. We applied 50- and 100 µM DC to Vero, MCF-7 and granulosa cells for 24 h. The percentage of mitotic cells in untreated Vero and MCF-7 cells was $0.9 \pm 0.17\%$ and $0.66 \pm 0.10\%$, respectively. For 50- and 100 µM DC, the results were $0.93 \pm 0.25\%$ and $1.02 \pm 0.23\%$, respectively, in Vero cells and $0.7 \pm 0.26\%$ and $0.9 \pm 0.1\%$, respectively, in MCF-7 cells. Vero and MCF-7 cells showed no significant difference in terms of mitotic cell index between the control and DC-treated groups (Fig. 3A and B). The number of mitotic cells was also comparable between the control ($0.01 \pm 0.01\%$) and DC-treated granulosa cells ($0.03 \pm 0.06\%$ and 0.07 ± 0.12 for 50 and 100 µM doses, respectively) (Fig. 3C).

Effects of DC on cell viability

As illustrated earlier, although DC inhibited cell proliferation as determined by cell proliferation assays and Ki-67 staining, the mitotic cell index demonstrated that those effects were not due to dynamics of mitotic cell division. To determine whether DC has a cytotoxic effect on proliferating cells, cell viability was determined next in 10, 50, 100 and 200 µM DC-treated Vero and MCF-7 cells for 24 or 48 h while 50 and 100 µM DC was applied to granulosa cells. The most significant decrease in Vero cell viability was found in the 100 and 200 µM groups at 24

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($P = 0.006$ and $P = 0.002$, respectively) and 48 h ($P = 0.003$ and $P = 0.002$, respectively) of drug exposure (Fig. 4A). Viability of MCF-7 cells was 91 ± 1% in control group, then significantly decreased to 87 ± 2% in 50 μM DC ($P = 0.016$), 79 ± 1% in 100 μM DC ($P < 0.001$) and 66 ± 5% in 200 μM DC ($P < 0.001$) doses after 24 h incubation. Cell viability was further reduced in the 48-h incubation groups, and significantly in 100 ($P < 0.001$) and 200 μM ($P < 0.001$) DC-treated groups (Fig. 4B). Granulosa cells showed no significant difference between the control and DC-treated groups in terms of cell viability at 24 and 48 h (Fig. 4C). These results indicated that DC reduces the viability of Vero and MCF-7 cells in a time- and dose-dependent manner, while the percentage of viable granulosa cells does not change following DC-treatment *in vitro*.

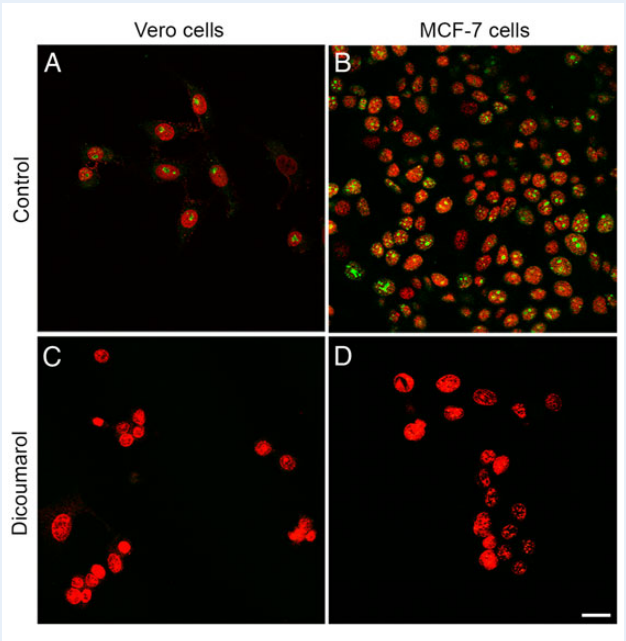

Figure 2 Ki-67 staining of Vero and MCF-7 cells. Ki-67 staining (green signal) was confined to nucleolar positivity in control Vero (**A**) and MCF-7 (**B**) cells. No DC-treated samples displayed any Ki-67 positivity (**C** and **D**). Representative images in C and D were taken from a 100 μM DC group. Red signal: Nuclei; Scale bar: 20 μm. Results were evaluated for three experimental replicates.

Determination of the apoptotic cell index

The apoptotic cell index was then determined using TUNEL assay to address the underlying cause of decreasing cell viability. We applied 50 and 100 μM DC to Vero, MCF-7 and granulosa cells for 24 h. The percentage of TUNEL$^+$ Vero cells was 1.05 ± 0.84% in the control group versus 13.33 ± 10.96% in 50 μM- and 18.66 ± 12.5% in 100 μM DC-treated ($P = 0.048$) groups (Fig. 5A). The number of TUNEL$^+$ MCF-7 cells was significantly higher in 50- (3.66 ± 1.52%) ($P = 0.018$) and 100 μM (9.5 ± 1.5%) ($P < 0.001$) DC-treated groups compared with the controls (0.4 ± 0.1%) (Fig. 5B). In contrast, no difference was noted between the control and DC-treated granulosa cells in TUNEL positivity (Fig. 5C).

Above results were confirmed with Annexin-V labelling in 100 μM DC-treated groups. Vero cells showed no significant difference between the control and 100 μM DC-treated groups in Annexin-V positivity (Fig. 5D). The number of Annexin-V$^+$ MCF-7 cells was higher ($P < 0.001$) in 100 μM DC-treated cells (36 ± 1%) versus controls (1 ± 1%) (Fig. 5E). There was no difference in the percentage of Annexin-V$^+$ granulosa cells in the control and 100 μM DC-treated groups (Fig. 5F). Both TUNEL and Annexin-V assays revealed that DC treatment increases the percentage of apoptosis in Vero and MCF-7 cells; however, it does not change the percentage of apoptotic granulosa cells *in vitro*.

Effects of DC on NQO1 expression

To determine whether the apoptotic effect of DC is related to NQO1, 100 μM DC was applied to Vero, MCF-7, and granulosa cells for 24 h. Confocal immunofluorescence and western blot analyses revealed no NQO1 expression in Vero cells (Fig. 6A and D), whereas both MCF-7 (Fig. 6B) and granulosa cells were found positive for NQO1 (Fig. 6C). However, no significant difference between the control and DC-treated MCF-7 cells was noted in NQO1 quantity (Fig. 6E). CTCF calculation revealed that expression levels of NQO1 were comparable between control and DC-treated granulosa cells (Fig. 6F). These results suggested that NQO1 might not be related to the adverse effects of DC on Vero and MCF-7 cells, as it was not expressed in both control and DC-treated

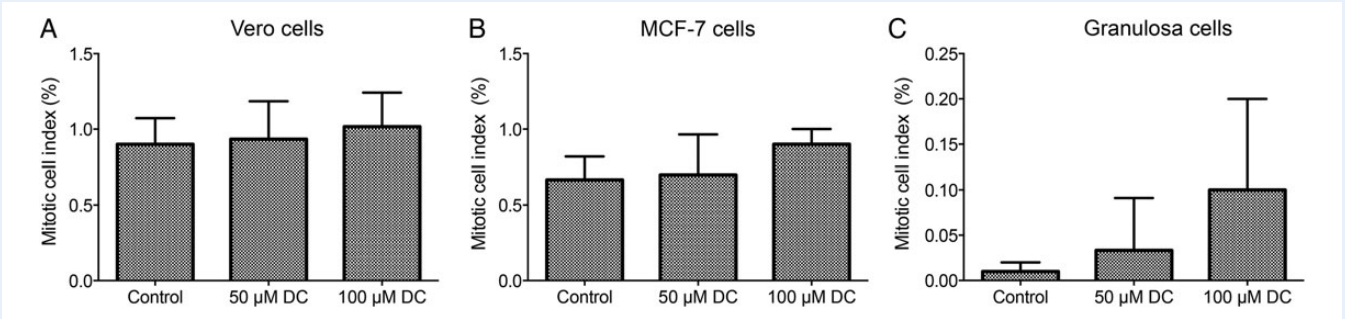

Figure 3 Mitotic indices of Vero, MCF-7 and granulosa cells. Mitotic cell indices of Vero (**A**), MCF-7 (**B**) and granulosa (**C**) cells after treatment with 50 and 100 μM DC. No cell types showed significant mitotic cell index difference between control and DC-treated groups. Results were shown as mean and standard deviation for three experimental replicates. Non-parametric analyses were performed with Chi-square (χ^2) test.

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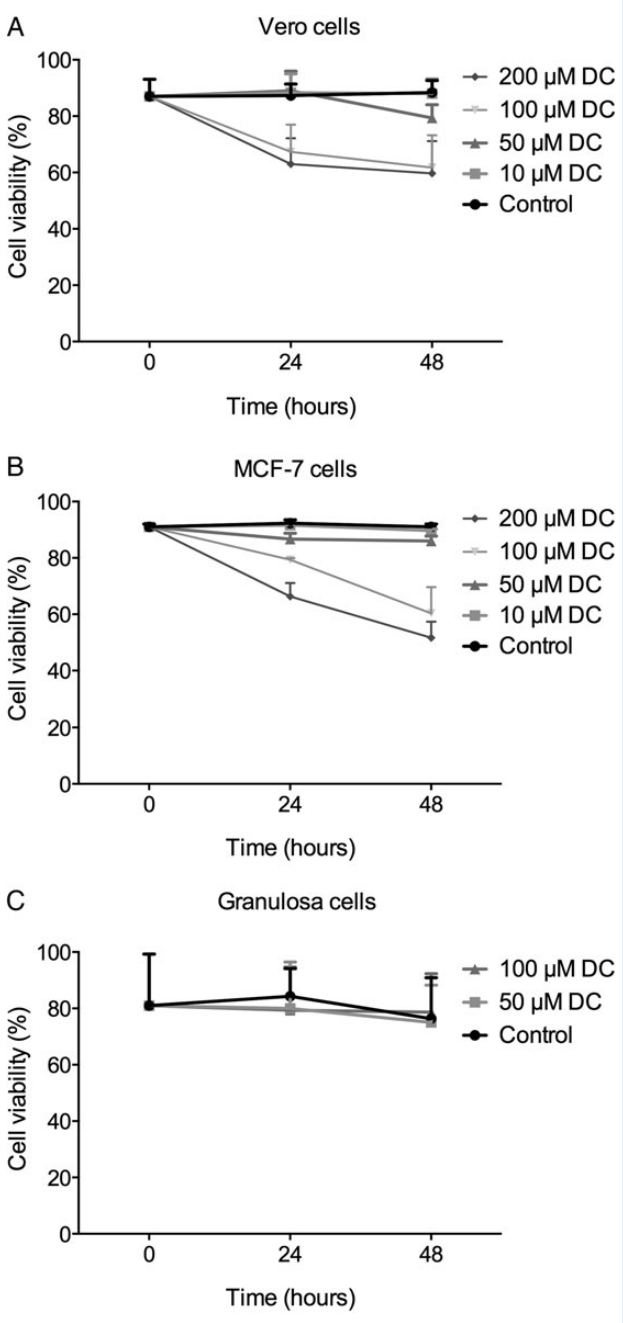

Figure 4 Cell viability analysis in DC-treated cells. Frequencies of cell viability in Vero (**A**), MCF-7 (**B**) and granulosa (**C**) cells for 24 and 48 h in control medium or in 10, 50, 100 and 200 μM DC incubations. The maximum decrease in cell viability was found in 100- and 200 μM groups after 24 ($P = 0.006$ and $P = 0.002$, respectively) and 48 h ($P = 0.003$ and $P = 0.002$, respectively) in Vero cells, in 50 μM ($P = 0.016$), in 100 μM ($P < 0.001$) and in 200 μM ($P < 0.001$) after 24 h and in 100- ($P < 0.001$) and 200 μM ($P < 0.001$) after 48 h in MCF-7 cells. In contrast, no cell viability difference was noted in granulosa cells. Results were shown as mean and standard deviation for three experimental replicates. Normally distributed data were analysed with one-way ANOVA and Fisher's LSD post-hoc tests.

Vero cells, and DC treatment did not alter the quantity of NQO1 in MCF-7 or in granulosa cells.

Effect of DC on meiotic spindle in comparison with paclitaxel

Based on previous reports (Jacobs *et al.*, 2003; Madari *et al.*, 2003) that DC has a microtubule stabilization effect similar to paclitaxel on the cell mitotic spindle, isolated M-II stage oocytes were either treated with 5 μM paclitaxel ($n = 80$) or 100 μM DC ($n = 70$) for 60 min *in vitro* to compare their mechanisms of action in terms of meiotic spindle integrity and chromosome distribution. Meiotic spindles of DC-treated oocytes were observed as the typical barrel shape, whereas the meiotic spindle microtubule mass was diminished and associated with scattered microtubule organizing centres (MTOCs) and displaced chromosomes in the paclitaxel-treated group (Fig. 7). When considered with our mitotic index results, these data demonstrated that DC and paclitaxel do not share the same mechanism of action in mouse oocytes.

Effects of DC on *in vitro* maturation of mouse oocytes

To screen the effects of DC on meiotic spindle formation during *in vitro* maturation, GV-stage oocytes were treated with 100 μM DC. Following 18 h of incubation, 57.7 ± 19.4% of untreated oocytes reached the M-II stage (Fig. 8A). A typical barrel-shaped meiotic spindle was observed in each M-II stage oocyte and chromosomes were aligned properly at the metaphase plate (not shown). DC-treated oocytes, either those incubated in maturation medium or DC-microinjected, showed no significant difference in terms of oocyte numbers that reached the M-II stage compared with the control group. Both meiotic spindle integrity and chromosome alignment were found to be normal (not shown).

The effect of DC on meiotic maturation was further analysed through evaluating the *in vitro* matured GV-stage oocytes following *in vivo* DC treatment. Both the control and *in vivo* DC-treated groups reached the M-II stage at a rate of 44.0 ± 19.0% and 51.0 ± 20.0%, respectively (Fig. 8B). There was no difference in the meiotic spindle and chromosome alignment when compared with the controls (not shown). These results revealed that oocyte maturation is not blocked following *in vitro* or *in vivo* DC treatment and DC does not show any detrimental effect on meiotic spindle or chromosomes in M-II stage oocytes.

Effects of *in vivo* DC treatment on mouse ovaries

Based on previous reports (Turkyilmaz *et al.*, 2008; Sonmezer and Ozkavukcu, 2009) that chemotherapy drugs affect the primordial follicles causing loss of fertility, ovaries from control and 32 mg/kg DC-treated mice were evaluated in terms of primordial follicles. The number of primordial follicles was comparable between the control (8.6 ± 1.5) and DC-treated (9.3 ± 2.9) groups (Fig. 9A). There was no significant difference in terms of TUNEL$^+$ granulosa cells between control and DC-treated primordial follicles (1.0 ± 2.1% and 3.2 ± 6.7%, respectively) ($P = 0.273$). TUNEL index was also comparable between groups as 0.1 ± 0.3% and 1.7 ± 3.0% in primary follicle ($P = 0.200$); 0.3 ± 0.2% and 0.3 ± 0.6% in secondary follicle ($P = 1.000$) and 1.5 ± 3.0% and 0.8 ± 1.1% in antral follicle ($P = 0.589$) in control and DC-treated

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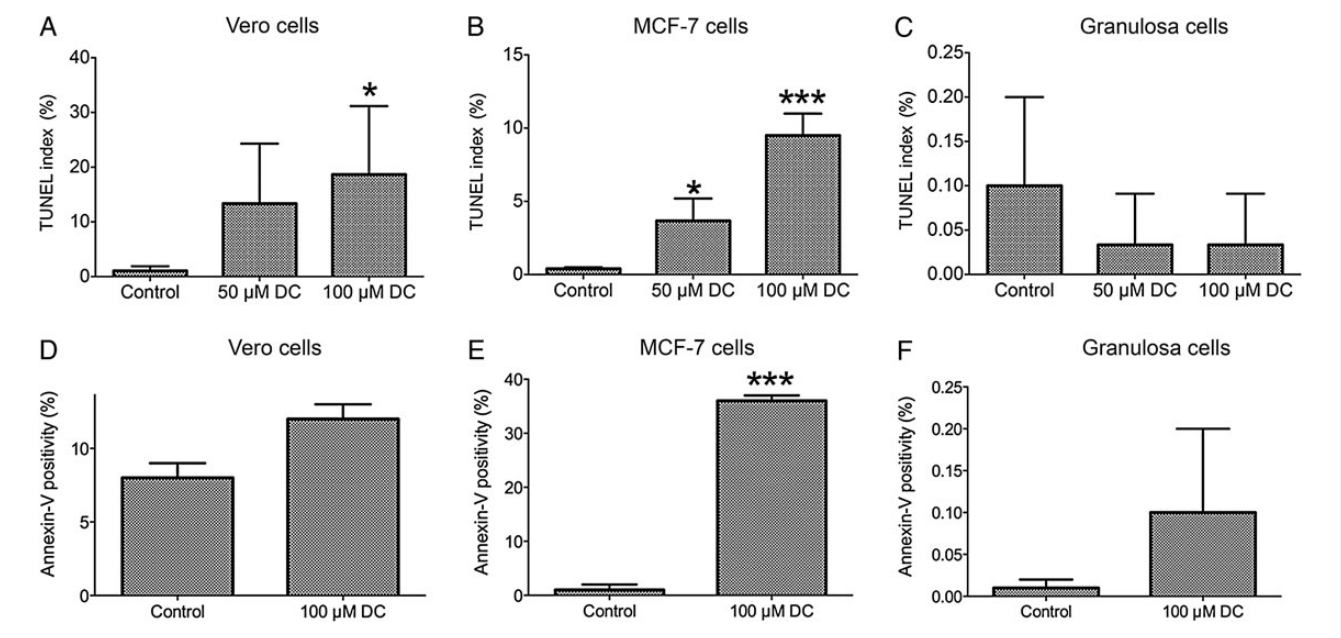

Figure 5 Apoptotic indices in DC-treated cells. Percentage of TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labelling) index (**A–C**) and Annexin-V positivity (**D–F**) in Vero (A, D), MCF-7 (B, E) and granulosa (C, F) cells. There was a significant difference between the control and 100 μM DC-treated Vero cells in terms of TUNEL positivity (A). The number of TUNEL$^+$ MCF-7 cells was significantly higher in 50- and 100 μM DC-treated groups compared with the controls (B). On the other hand, no difference was noted between the control and DC-treated granulosa cells in TUNEL positivity (C). The number of Annexin-V$^+$ Vero cells was higher in 100 μM DC-treated group compared with the controls (D). The number of Annexin-V$^+$ MCF-7 cells was significantly higher in 100 μM DC-treated cells versus controls (E). There was no difference between the control and 100 μM DC-treated granulosa cells in terms of Annexin-V$^+$ positivity (F). *$P < 0.05$, ***$P < 0.001$ Results were shown as mean and standard deviation for three experimental replicates. Non-parametric analyses were performed with Chi-square (χ^2) test.

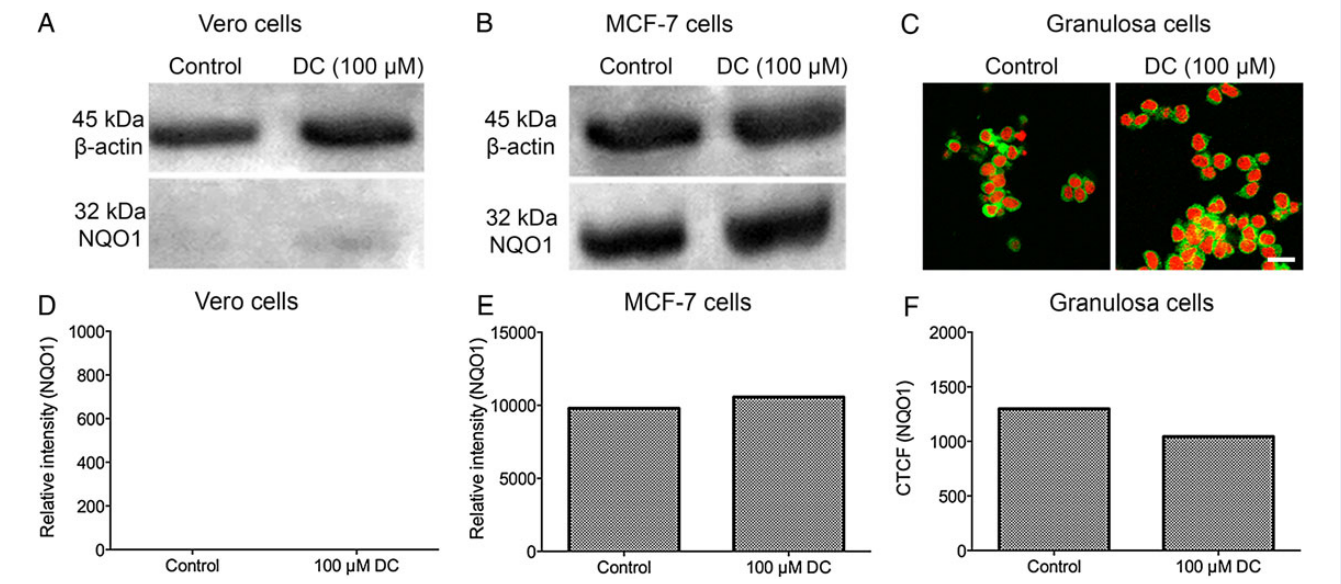

Figure 6 NQO1 in DC-treated cells. NQO1 [NAD(P)H Quinone Oxidoreductase 1] quantity in Vero (**A**, **D**), MCF-7 (**B**, **E**) and granulosa (**C**, **F**) cells in control and 24 h DC (100 μM) groups. Western blot analyses revealed no NQO1 expression in Vero cells (A, D), whereas MCF-7 cells were found positive (B, E). However, no significant difference between the control and DC-treated MCF-7 cells was noted in NQO1 quantity (E). Confocal immunofluorescence observations of granulosa cells provided strong positivity for NQO1 (green signal in C). CTCF calculation revealed that expression levels of NQO1 were comparable between control and DC-treated granulosa cells (F). Red signal in C: nuclei; scale bar: 10 μm. Results were shown for three experimental replicates. Normally distributed data were analysed with one-way ANOVA and Fisher's LSD post-hoc tests.

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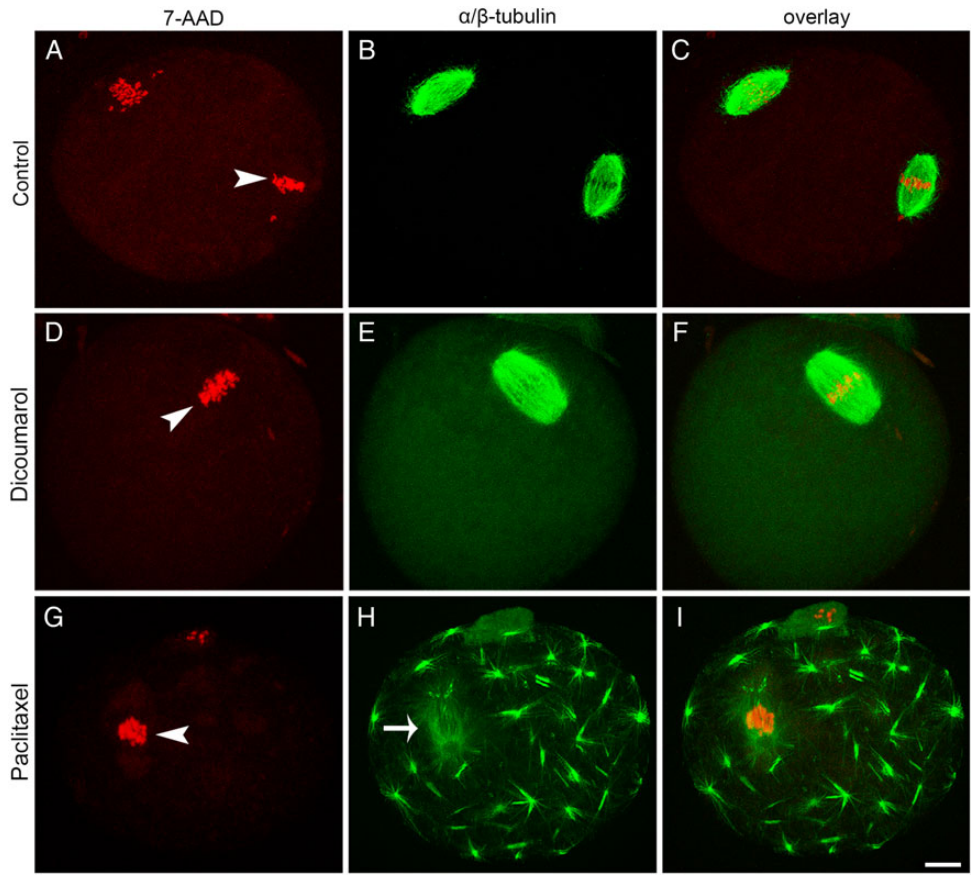

Figure 7 Chromosome and meiotic spindle patterns in DC/paclitaxel-treated oocytes during *in vitro* maturation. *In vitro* matured Metaphase-II (M-II) stage oocytes in control (**A**–**C**), DC (100 μM) (**D**–**F**) and paclitaxel-containing (5 μM) (**G**–**I**) medium for 1 h. DC-treated M-II stage oocytes did not display any spindle microtubule disruption and chromosome organization (arrowhead in D) compared with the controls (arrowhead in A). In contrast, paclitaxel caused a severe disruption of MTOCs (Microtubule Organizing Centres as scattered throughout the ooplasm and a decrease in the spindle microtubule intensity (arrow in H) with a dissociated chromosome set (arrowhead in G). Green signal: microtubules. Red signal: meiotic chromosomes with 7-AAD (7-aminoactinomycine D). Scale bar: 10 μm. Results were evaluated for three biologic replicates.

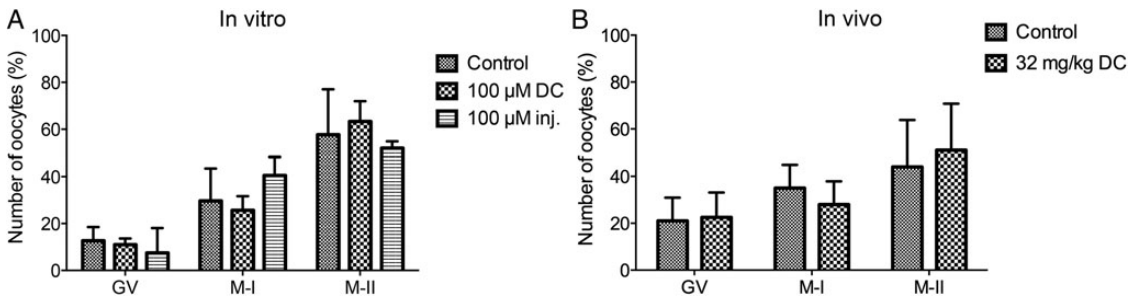

Figure 8 *In vitro* maturation kinetics of oocytes obtained from DC-administered animals. *In vitro* maturation of DC-incubated oocytes (100 μM) (**A**) and oocytes after animals were treated *in vivo* with DC (32 mg/kg) (**B**). No significant difference was noted between control and DC-incubated or DC-injected oocytes regarding the maturation resumption to metaphase-I (M-I) and M-II (A). Similar to *in vitro* results, no difference was noted in *in vitro* matured oocytes after isolated from *in vivo* DC-treated animals (B). GV: Germinal Vesicle. Results were shown as mean and standard deviation for three biologic replicates. Non-parametric analyses were performed with Chi-square (χ^2) test.

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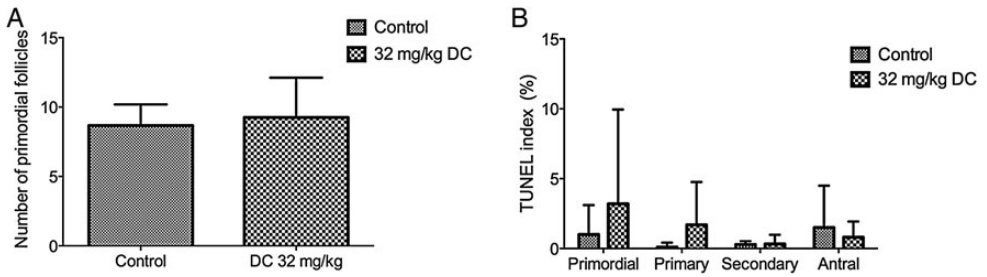

Figure 9 Effects of DC on ovarian follicle development and apoptosis. The number of primordial follicles (**A**) and percentage of TUNEL index (**B**) in control and 32 mg/kg DC-treated mouse ovaries. The number of primordial follicles was comparable between the control and DC-treated groups (A). There was no significant difference in terms of TUNEL^{+} granulosa cells between control and DC-treated primordial, primary, secondary follicles and antral follicles (B). Results were shown as mean and standard deviation for three biologic replicates. Non-parametric analyses were performed with Chi-square (χ^2) test.

groups, respectively (Fig. 9B). Corpus luteum displayed high TUNEL positivity in all ovarian sections and was used as an internal positive control. These data showed that DC treatment has no adverse effects on mouse ovaries regarding the number of primordial follicles or the ratio of apoptosis in primordial, primary, secondary and antral follicles.

Discussion

Cell division-targeted anticancer agents are expected to inhibit cell proliferation and/or induce cell death without damaging healthy cells. DC was first suggested as an anticancer agent in 1985 because it enhanced the anti-proliferative effects of mitomycin-C in a mouse model of experimental pancreatic cancer (Keyes *et al.*, 1985). The cytotoxic effects of DC have since been demonstrated in various studies. Brar *et al.* showed that proliferation of human melanoma cells decreased with increased doses of DC (Brar *et al.*, 2001). Moreover, DC supressed proliferation (Cullen *et al.*, 2003; Lewis *et al.*, 2004), decreased viability (Cullen *et al.*, 2003) and induced apoptosis (Lewis *et al.*, 2004) of human pancreatic cancer cells, in a time- and dose-dependent manner. DC has also been reported to alter anticancer effects of several chemotherapeutics, including cisplatin (Watanabe *et al.*, 2006), gemcitabine (Buranrat *et al.*, 2010) and doxorubicin (Matsui *et al.*, 2010) in prostate, liver and uroepithelial cancer cells, respectively. In the present study, DC's anti-proliferative and apoptotic effects were demonstrated in two well-studied epithelial noncancerous (i.e. Vero) and cancerous cell lines (i.e. MCF-7). Proliferation was significantly supressed in 100- and 200 μM DC-treated Vero cells when incubated for 24 and 48 h whereas suppression of MCF-7 cell proliferation was detected at a 10 μM DC dose at both time points. Both Vero and MCF-7 cell viability decreased significantly at 100- and 200 μM DC doses. Furthermore, DC treatment induced apoptosis in both cell lines at 50- and 100 μM doses. Brar *et al.* demonstrated that DC treatment had no adverse effects on rat airway myocytes (Brar *et al.*, 1999). Likewise, 50- or 100 μM DC treatment of granulosa cells had no effect on cell number, viability or apoptosis in our study. Our findings were consistent with the literature, as distinct DC doses had varying degrees of detrimental effects on different cell types. Therefore, the anti-proliferative and apoptotic effects were time- and dose-dependent, similar to previous findings (Brar *et al.*, 2001; Cullen *et al.*, 2003; Lewis *et al.*, 2004).

The anticancer effect of DC has recently been associated with the inhibition of NQO1, an oxidoreductase responsible for the reduction of quinones to hydroquinones. Various cancer cells express high levels of NQO1 compared with normal tissue (Begleiter and Fourie, 2004). It has been demonstrated that DC treatment increases ROS production in NQO1-positive pancreas cancer cells (Lewis *et al.*, 2004). Buranrat *et al.* reported that with DC treatment ROS levels increase in NQO1-expressing liver cancer cells, while ROS remain unchanged in NQO1-negative cells (Buranrat *et al.*, 2010). In an *in vitro* study, exposure of uroepithelial cancer cells to DC resulted in p53 instability and PARP [(poly (ADP-ribose) polymerase] activation, which lead to apoptosis (Matsui *et al.*, 2010). In our study, we have demonstrated that Vero cells do not express NQO1, while MCF-7 and granulosa cells were NQO1 positive. There was no significant difference between control and DC-treated groups in terms of NQO1 distribution and quantity. Likewise, Du *et al.* demonstrated no difference in the cytotoxic effect of DC in NQO1-positive and NQO1-negative breast cancer cells and ROS production occurred through a distinct pathway (Du *et al.*, 2006). Thus, DC's anti-proliferative and apoptotic effects may not be related to the inhibition of NQO1.

It has been demonstrated that DC binds to tubulin *in vitro* and blocks the first division of sea urchin embryos in a dose-dependent manner (Madari *et al.*, 2003). Jacobs *et al.* reported that DC delays microtubule depolymerization, thus may act like a microtubule stabilizer (Jacobs *et al.*, 2003). In the present study, this hypothesis was rejected for the following reasons; (i) No pause in mitosis was detected in DC-treated Vero or MCF-7 cells. (ii) DC-treated GV-stage oocytes completed meiosis in a given time in culture. In our study, Vero and MCF-7 cells were considered as rapidly proliferating cells, whereas granulosa cell proliferation was remarkably slow. The primary effect of DC in cell cycle progression may not be associated with the M-phase dynamics of proliferating cells. Follow-up studies that identify the molecular mechanisms that regulate this relationship will provide an important contribution to the literature.

To the best of our knowledge, the present study evaluates the effect of DC on ovarian tissue at cellular level for the first time. Infertility is one of the most common adverse long-term effects that results from widely used chemotherapeutics, together with radiation therapy (Sonmezer and Ozkavukcu, 2009). For instance, treatment of childhood cancers with a combination of procarbazine and busulphan induces irreversible early menopause (Brachet *et al.*, 2007). It has also been suggested that

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childhood cancer treatment with procarbazine, chlorambucil, vinblastine or prednisolone may result in gonadal dysfunction (Mackie *et al.*, 1996). Our findings revealed that DC does not block mouse oocyte maturation *in vitro* or *in vivo*. It is known that defects in the meiotic spindle may result in chromosomal abnormalities that include non-disjunction, chromosomal scattering and aneuploidy (Can *et al.*, 2003). In the present study, none of the DC-treated oocytes that reached the M-II stage showed damaged meiotic spindle or scattered chromosomes. On the other hand, paclitaxel treated M-II stage oocytes were characterized by a perished spindle structure, dispersed chromosomes and scattered MTOCs throughout the cytoplasm. Although few studies suggest that DC is a microtubule stabilizing agent synergistic with paclitaxel (Madari *et al.*, 2003), these findings, together with our mitotic index results, show that DC and paclitaxel do not share the same mechanism of action. It has been reported that treatment of breast cancer with cyclophosphamide enhanced blood FSH levels and led to amenorrhea (Koyama *et al.*, 1977). In the same study, ovarian biopsies showed no sign of follicles or oocytes. We have demonstrated that DC does not induce apoptosis either in granulosa cells *in vitro* or in ovarian tissue *in vivo*, which suggests that DC is a good alternative as an anticancer agent, regarding fertility preservation. On the other hand, the possibility that DC may potentiate ovarian toxicity when combined with traditional chemotherapeutic agents, such as mitomycin-C (Keyes *et al.*, 1985), cisplatin (Watanabe *et al.*, 2006), gemcitabine (Matsui *et al.*, 2010) and doxorubicin (Matsui *et al.*, 2010), must be taken in to account, as DC alters their anticancer effects in particular cancer cells.

In conclusion, the present study demonstrated that DC inhibited proliferation, decreased viability and induced apoptosis in a time- and dose-dependent manner in rapidly proliferating cells, without stabilization of microtubules or inhibition of NQO1. Granulosa cells and isolated oocytes both *in vitro* and *in vivo* were not adversely affected by DC treatment. Based on our findings, we suggest that DC is a potentially useful anticancer agent and may contribute to fertility preservation in cancer treatment through a mechanism yet to be determined.

Supplementary Data

Supplementary data are available at http://molehr.oxfordjournals.org/.

Acknowledgement

This work includes data from dissertation thesis entitled 'Effects of dicoumarol on mitotic and meiotic cells as an anticancer agent' by DA, 2014.

Authors' roles

All authors played a role in experimental design and data acquisition. D.A., O.C. and Z.C. carried out experimental procedures. The manuscript was prepared by D.A., O.C. and A.C. D.A. and A.C. submitted the manuscript.

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Conflict of interest

None declared.

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