Toxicology in Vitro 24 (2010) 108–115

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

The effect of glucocorticoids on mouse oocyte in vitro maturation and subsequent fertilization and embryo development

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ARTICLE INFO

Article history:
Received 5 September 2008
Accepted 26 August 2009
Available online 3 September 2009

Keywords:
Corticosterone
Cortisol
Dexamethasone
ERK-1/2
MAP kinase
Oocyte
Maturation
Fertilization
Embryo development

ABSTRACT

Increased glucocorticoid levels, due to medical therapy or stress-related, may affect reproduction via the hypothalamus–pituitary-axis or directly at the oocyte level. We examined the effects of natural (corticosterone) or synthetic (dexamethasone) glucocorticoids on mouse oocyte maturation and underlying changes in extracellular signal-regulated kinase (ERK) phosphorylation patterns. Fertilization and progression up to the blastocyst stage were also evaluated. Oocytes were exposed to corticosterone or dexamethasone (0, 0.25, 2.5, 25 or 250 μM) for 17 h during in vitro maturation. After maturation, ERK-1/2 activation in oocytes was assessed by SDS–PAGE and immunoblotting, and fertilization and developmental capacity were examined in vitro. Corticosterone exposure during oocyte maturation significantly decreased progression to metaphase II, fertilization and embryo development at the highest concentration. Corticosterone caused a concentration-dependent inhibition of ERK-1/2 activation, with the highest concentration resulting in considerable inhibition of oocyte ERK-1/2 phosphorylation and no blastocyst development. In contrast, dexamethasone had no effect on maturation, fertilization and cleavage, and no effect was seen on ERK-1/2 phosphorylation. Based on these in vitro findings, high glucocorticoid levels may have consequences for subsequent development, although a short exposure to physiologic or stress-related glucocorticoid levels may not represent a hazard to meiosis progression of the oocyte.

1. Introduction

Glucocorticoids are steroid hormones released from the adrenal cortex to maintain homeostasis. In stressful situations high amounts of glucocorticoids are released through activation of the hypothalamic–pituitary–adrenal axis. It is generally accepted that stress influences reproduction, with chronic stress negatively affecting reproductive performance (Brann and Mahesh, 1991; Moberg, 1991; Sapolsky et al., 2000; Tilbrook et al., 2000). Wild animals are very sensitive to the stressful conditions that they experience when maintained in captivity and this has been considered a limiting factor in their reproduction (Hutchins et al., 1996; Swaisgood et al., 2006; Zhang et al., 2004). Moreover, disruptive effects of stress on assisted reproduction technologies in wild animals have been reported (Dorn, 1995; Fernández-Arias et al., 2000; Ledda et al., 1995). Elevated corticosteroid levels during stressful conditions, or after medical therapy employing glucocorticoids, could have a negative impact on reproduction. Finally, stress could also reduce the efficacy of fertility treatments in humans (Campagne, 2006; Cwikel et al., 2004; Hjollund et al., 1999).

Females are more sensitive to the disturbances of stress than males, with reproduction being affected by glucocorticoids mainly through the hypothalamus–pituitary-axis (Ferin, 2006), but also at the gonadal level, modulating steroidogenesis and/or oogenesis (Ben-Rafael et al., 1988; Huang and Li, 2001; Michael et al., 1993; Michael and Cooke, 1994). Glucocorticoids reach the ovary through the blood stream exerting their actions through glucocorticoid receptors present on ovarian cells (Schreiber et al., 1982; Tetsuka et al., 1999) and they are regulated by 11-hydroxysteroid dehydrogenase (11β-HSD) isozymes (Goodman, 2003; Michael et al., 2003). Two 11 β-HSD isozymes catalyze the interconversion of biologically active corticosteroids (cortisol/corticosterone) and inactive metabolites (cortisone/11-dehydrocorticosterone) (Michael et al., 2003).

An altered follicular environment after exposure to high levels of glucocorticoids during oocyte maturation could affect the future competence of the oocyte. However, studies on the direct effect of glucocorticoids on in vitro oocyte maturation have reported conflicting results in pig and mouse oocytes (Andersen, 2003; Yang

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doi:10.1016/j.tiv.2009.08.025

et al., 1999). An inhibitory effect was noticed on meiosis resumption after incubation of pig oocytes in the presence of cortisol and dexamethasone, and this action was mediated through the glucocorticoid receptor (Yang et al., 1999). On the other hand, no effect was found on spontaneous or induced maturation in mouse oocytes exposed to cortisol or dexamethasone during culture (Andersen, 2003). A recent report has undertaken a thorough study of the effect of dexamethasone on mouse ovarian function and early embryo development (Van Merris et al., 2007) and results revealed that high concentrations of dexamethasone affected maturation and blastocyst development.

Little is known about the underlying mechanisms of glucocorticoid action on oocytes during in vitro maturation. The inhibitory action of glucocorticoids on pig oocytes (Yang et al., 1999) was attributed, at least partially, to the reduction in the maturation/metaphase-promoting factor (MPF) (Chen et al., 2000). MPF and mitogen-activated protein kinases (MAPKs) are the principal regulatory systems driving the oocyte meiotic cell cycle (Abrieu et al., 2001; Fan and Sun, 2004; Liang et al., 2007; Motlik et al., 1998). MAPKs become activated by phosphorylation of threonine and tyrosine residues (Pearson et al., 2001). The extracellular signal-regulated kinases ERK-1 and ERK-2 are members of the MAPK family activated during oocyte maturation (Sun et al., 2002; Verlhac et al., 1993, 1994). The microtubule organization and chromatin condensation that takes place during oocyte maturation is associated with the activity of MAPK (Fan and Sun, 2004; Verlhac et al., 1994). Thus, although there is evidence suggesting that glucocorticoids can affect nuclear maturation through inhibition of MPF (Chen et al., 2000), it is not known whether glucocorticoids may alter activation of MAPK during oocyte maturation.

It should be noted that previous studies on mouse oocytes examined the effect of cortisol on in vitro maturation (Andersen, 2003) or that of the synthetic glucocorticoid dexamethasone on in vitro maturation (Andersen, 2003), or on folliculogenesis, oogenesis and embryo development (Van Merris et al., 2007). However, in rodents, the major glucocorticoid is corticosterone and the effect of this endogenous glucocorticoid on oocyte maturation and subsequent development has not been examined.

Dexamethasone is a long-acting synthetic glucocorticoid, which shares the structure of the natural glucocorticoid, but has a higher potency. It circulates mainly in the unbound form or bound to albumin and it is minimally bound to transcortin (corticosteroid-binding globulin, CBG) (Magiakou and Chrousos, 1994; Pugeat et al., 1981) unlike the natural glucocorticoids which are mostly bound to transcortin with only a small proportion free and biologically active (Goodman, 2003). Thus, dexamethasone is commonly used to increase the effects of the natural glucocorticoids.

The present study was designed to examine the effects of the natural and a synthetic glucocorticoid (corticosterone and dexamethasone, respectively) on maturation of mouse oocytes and to uncover possible actions on underlying mechanisms of MAPK activation. Although nuclear maturation can be completed successfully, several events globally referred to as cytoplasmic maturation are required for completion of development following fertilization. For this reason, we have also examined potential effects of the glucocorticoids on oocyte maturation that would reflect on fertilization or subsequent development. The aims of the present study were thus to: (a) assess the effects of corticosterone and dexamethasone on in vitro maturation of mouse oocytes, (b) evaluate whether corticosterone and dexamethasone affected MAPK phosphorylation during oocyte maturation, and (c) assess the capacity of oocytes exposed to corticosterone or dexamethasone during maturation to undergo fertilization and subsequent embryonic development up to the blastocyst stage.

2. Materials and methods

2.1. Reagents

Chemicals used in the present study were obtained from Sigma Chemical Co. (Madrid, Spain), unless indicated otherwise.

2.2. Animals

All the animal experiments performed in this study were conducted in accordance with the Spanish Animal Protection Regulation, RD1201/2005 which, in turn, conforms to European Union Regulation 2003/65.

B6D2F1 mice were used. Animals were maintained on a 14:10 h light:darkness photoperiod in a temperature-controlled room at 22–24 °C. Food and water was provided ad libitum.

2.3. Collection of oocytes and in vitro maturation (IVM)

To stimulate follicular development, females received an intraperitoneal injection of 5 IU eCG (Folligon, Intervet, Madrid, Spain) at 10–12 weeks of age. After 48 h of hormone administration, females were sacrificed by cervical dislocation and their ovaries immediately excised and placed in warm M2 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS, Gibco, Madrid, Spain) and antibiotics (penicillin G and streptomycin sulphate, 50 μg/ml each). Cumulus-oocyte complexes (COCs) were released by follicular puncturing with the aid of 30G needles attached to disposable syringes. After 3–4 washes in M2, only immature oocytes surrounded by compact cumulus cells were selected, rinsed once in maturation medium [Minimum Essential Medium-alpha with Earle salts (MEM-alpha, Gibco) plus 10% (v/v) heat-inactivated FCS and penicillin G and streptomycin sulphate, 50 μg/ml each] and finally placed in 4-well culture dishes (Nunclon, Nalgene, Nunc International, Roskilde, Denmark) containing 500 μl of maturation medium in each well. For all the experiments, groups of approximately 15–25 COCs per well were cultured at 37 °C under 5% CO_2 in air and maximum humidity for 17 h. Mineral oil was not used to overlay the media to avoid possible absorption of liphophylic compounds (Reinsberg et al., 2004; Shimada et al., 2002; Waterman and Wall, 1988). Dexamethasone (D-1159) or corticosterone (C-2505) were added at the beginning of culture as indicated in Section 2.9.

2.4. In vitro fertilization (IVF)

In each experiment, one 12- to 16-week-old B6D2F1 male was sacrificed by cervical dislocation. Caudae epididymides and vasa deferentia were excised and placed in a dish (35 × 10 mm) containing 1 ml of pre-equilibrated human tubal fluid medium (HTF) covered by mineral oil, as suggested by The Jackson Laboratory IVF protocol (http://cryo.jax.org/ivf.html). With the aid of 30G needles, the tissue was minced and incubated for 10 min at 37 °C in 5% CO_2/air to allow the spermatozoa to swim-out. Subsequently, the minced tissue was removed and the sperm suspension further incubated up to 30–40 min until used for co-incubation with the oocytes. At the end of IVM, oocytes were washed twice in HTF medium before incubation with spermatozoa. The final concentration of spermatozoa used during IVF was 1×10^6 motile spermatozoa/ml. Gametes were co-incubated in 4-well culture dishes in 500 μl of HTF medium without mineral oil, for 4–6 h at 37 °C under 5% CO_2 in air.

2.5. In vitro culture

After gamete co-incubation, presumptive zygotes were washed in KSOM culture medium supplemented with 1% (v/v) non-essential

and 2% (v/v) essential amino acids (Gibco) (KSOMaa) (Nagy et al., 2003) and gently pipetted to remove spermatozoa and attached cumulus cells. Presumptive zygotes were then transferred to 500 μl of KSOMaa medium and cultured at 37 °C under 5% CO_2 in air with maximum humidity. The medium was renewed after 44–48 h of culture. At this point, one-cell (non-cleaved) oocytes were removed and stained (see below) whereas cleaving embryos were further cultured in fresh KSOMaa until day 5. Day of fertilization was considered as day 0. Blastocyst development was monitored on day 5 and blastocysts were classified as early blastocyst (Be), blastocyst (Bl), expanded blastocyst (Bexp) or hatching blastocyst (Bhing).

2.6. Evaluation of meiosis progression

The resumption and progression through meiosis was evaluated retrospectively after in vitro fertilization and culture. Non-cleaved (one-cell) oocytes were stained with Hoechst 33342 after 44–48 h of culture. Hoechst was dissolved in PBS and further diluted (1:10 v/v) in glycerol to reach a final concentration of 10 μg/ml (staining and mounting medium). Oocytes were included in small drops of the staining and mounting medium and they were immediately mounted onto slides under a coverslip supported by a mix of vaseline–paraffin (10: 0.7, w/w). Preparations were kept refrigerated until evaluation. Oocytes were visualized by using a microscope equipped with epifluorescence to confirm maturation status and/or fertilization. Fluorescence of Hoechst was observed using an Hg excitation beam and a filter set (UV-2A Nikon) consisting of a UV330–380 excitation filter, a DM400 chromatic beam splitter and a 420 barrier filter. Maturation rate was calculated as the number of oocytes that reached metaphase II (MII) over the total number of oocytes incubated. Fertilization rate was the number of fertilized oocytes over mature oocytes. Oocytes were considered to be fertilized when the presence of $\geqslant 2$ pronuclei and the polar bodies were identified. Cleavage was expressed as the number of embryos over fertilized oocytes and number of embryos over matured oocytes.

2.7. SDS–PAGE and immunoblotting

After IVM, oocytes were released from their surrounding cumulus cells by gentle pipetting with the aid of a narrow-bore glass pipette and washed five times in PBS with 0.1% (w/v) polyvinylpirrolidone (PVP). For each treatment, 30 denuded oocytes were collected and transferred in 5 μl of PBS plus PVP to centrifuge tubes, frozen immediately and stored at −80 °C until assays were performed. For analyses of kinase activation, oocytes were lysed in 5 μl of double-strength SDS sample buffer containing 5% 2- β-mercaptoethanol, boiled for 5 min and centrifuged for 2 min at 13,000 rpm. The supernatant containing soluble proteins was divided in two equal parts and each was separated in a 9% SDS–PAGE gel with 100:1 acrylamide:bisacrylamide. Each gel was transferred onto polyvinylidene fluoride membranes (Immobilon-P, Millipore, Badford, MA). Membranes were blocked for 1 h in 5% non-fat dry milk in Tris-buffered saline pH 7.4 with 0.05% Tween-20 (TTBS). One blot was incubated overnight in 5% BSA TTBS with anti-phospho-p44/42 MAP kinase antibody (#9101, dilution 1:1000; Cell Signalling, Barcelona, Spain) at 4 °C. The other blot was incubated for 1 h in 5% milk solution-TTBS with anti-total ERK rabbit polyclonal antibody (sc-94, dilution 1:1000; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Blots were then incubated for 1 h with secondary anti-rabbit, horseradish peroxidase-linked Ig (dilutions of 1:2000 or 1:5000 for phospho-ERK or total ERK detection, respectively; Amersham, Madrid, Spain) in 5% milk solution in TTBS. After being washed at least five times for at least 5 min per wash in TTBS, the blots were subsequently visualized by enhanced chemiluminescence (ECL) Western blotting detection reagent (RPN2109, Amersham), and X-ray film, following the instructions given by the manufacturer. The visualized bands were quantified by densitometry using NIH-Image J Sofware (http://rsb.info.nih.gov/ij/). The level of ERK activation of each treatment point was measured and corrected using total ERK values (i.e. ratio of activated p-ERK to total ERK). The p-ERK/total ERK ratio in oocytes after 24 h of IVM (controls) was arbitrarily set to 100% and values in experimental groups were expressed relative to this value. Experiments with each glucocorticoid were performed at least twice.

2.8. Statistical analysis

Data are expressed as percentages, and they were subjected to arcsine transformation when required. The data were analyzed using one-way ANOVA and, when significant differences were found, comparison between treatments was made using either the *post hoc* Dunnett's or Tukey tests. The Kruskal–Wallis ANOVA and Mann–Whitney *U*-tests were used when parametric assumptions were not fulfilled. Results are expressed as mean ± SEM. Probability values of less than 0.05 were considered significant. Statistical analyses were performed using Statistica (Statsoft, Inc. 2001. STATISTICA, data analysis software system, version 6; www.statsoft.com).

2.9. Experimental design

Corticosterone was solubilized in dimethyl sulfoxide (DMSO; D-2650) and dexamethasone was dissolved in milli-Q grade water. Stock solutions of 0.05, 0.5, 5 and 50 mM concentrations were prepared and stored at −20 °C until the day of use. The day of the experiment each stock was further diluted in a final volume of 500 μl of culture media to reach the desired concentrations. The final concentration of DMSO (0.5%, v/v) in culture media was the same in all treatment groups. An additional treatment without corticosterone, but with the same volume of DMSO was included as control group.

Corticosterone or dexamethasone were added at increasing concentrations (0, 0.25, 2.5, 25 and 250 μM) to incubation media during 17 h of in vitro maturation. After maturation, a sub-sample of oocytes was denuded, proteins extracted, separated and quantified after Western blotting; this was repeated at least twice for each range of glucocorticoid concentrations. Another sub-sample of oocytes was co-incubated with spermatozoa to assess the ability of the glucocorticoid-treated oocytes to undergo fertilization and progress in culture up to the blastocyst stage. The total numbers of degenerated oocytes after maturation, fertilization and after 44–48 h in culture were recorded. Experiments were carried out six times for corticosterone. For dexamethasone, experiments were repeated on seven independent occasions.

3. Results

3.1. Effects of corticosterone on in vitro maturation, fertilization and culture of mouse oocytes

Significant differences between treatments were found in maturation rate when oocytes were incubated in the presence of different concentrations of corticosterone ($F_{5,30} = 11.5$; $P < 0.001$). *Post hoc* analysis showed that the progression of oocytes to MII did not differ between controls (either with or without DMSO) or groups treated with 0.25, 2.5 or 25 μM corticosterone ($P > 0.05$), with about 80% of oocytes progressing to MII (Table 1). However, maturation rate was inhibited to almost half (45%) when oocytes were exposed to 250 μM corticosterone ($P < 0.001$) (Table 1). When

Table 1
Effect of exposure to increasing concentrations of corticosterone during in vitro mouse oocyte maturation on progression to metaphase II and subsequent fertilization and embryo development.

Treatment	Maturation rate, % (No. MII/no. oocytes in culture)	Fertilization rate, % (No. fertilized/no. matured)	Cleavage, % (No. cleaved/no. fertilized oocytes)	Overall cleavage, % (No. cleaved/no. mature oocytes)
Control (No DMSO)	86.3 ± 3.8[a] (95/108)	88.9 ± 4.7[a] (84/95)	95.7 ± 1.9[a] (81/84)	84.9 ± 3.2[a] (81/95)
Control (DMSO)	88.8 ± 4.0[a] (100/112)	91.6 ± 1.9[a] (91/100)	91.2 ± 0.8[a] (83/91)	83.5 ± 1.7[a] (83/100)
Corticosterone 0.25 μM	79.2 ± 3.3[a] (86/109)	92.7 ± 4.8[a] (78/86)	91.4 ± 2.4[a] (71/78)	84.6 ± 4.7[a] (71/86)
Corticosterone 2.5 μM	84.6 ± 5.4[a] (89/107)	90.5 ± 3.8[a] (79/89)	89.1 ± 3.5[a] (70/79)	80.9 ± 5.3[a] (70/89)
Corticosterone 25 μM	81.7 ± 3.6[a] (86/104)	81.2 ± 7.7[ab] (69/86)	81.9 ± 4.3[a] (44/69)	66.9 ± 8.4[a] (44/86)
Corticosterone 250 μM	45.3 ± 7.4[b] (48/106)	59.7 ± 10.7[b] (26/48)	42.0 ± 9.2[b] (10/26)	24.8 ± 7.2[b] (10/48)

Maturation rate is calculated as the number of oocytes in metaphase II (MII) over the total number of oocytes incubated. Fertilization rate is the number of fertilized oocytes over matured oocytes. Cleavage is expressed over fertilized oocytes and over matured oocytes. The data were analyzed using ANOVA. When significant differences were found, comparison between treatments was made using the *post hoc* Dunnett and Tukey tests. Different letters within columns denote $P < 0.05$. Data (percentages) are mean ± SEM, of six independent experiments.

cumulus expansion was subjectively assessed after maturation, it was found that in the group of oocytes exposed to 250 μM corticosterone there was only a limited expansion restricted only to the edge of the cumulus layer, or absence of expansion.

We found significant differences between the proportion of fertilized oocytes when oocytes were pre-exposed to corticosterone during maturation ($F_{5,30} = 4.0$; $P < 0.01$) (Table 1). Corticosterone added at 250 μM to media during IVM decreased the ability of mature oocytes to undergo fertilization when compared to controls ($P < 0.05$) or corticosterone at 0.25 ($P < 0.01$) or 2.5 μM ($P < 0.05$) (Table 1).

Cleavage, expressed as the number of cleaved oocytes over fertilized or matured ones, decreased as corticosterone concentration increased during IVM ($F_{5,30} = 12.9$; $P < 0.001$ and $F_{5,30} = 11.1$; $P < 0.001$, respectively). Significant differences were found for oocytes that were matured in the presence of 250 μM corticosterone; this concentration clearly hampered the developmental capacity of fertilized oocytes (Table 1).

Dead oocytes, based on their morphology after maturation, fertilization or after 48 h in culture, were regarded as degenerated. The proportion of degenerated oocytes was not different between controls and up to 25 μM corticosterone (data not shown). However, there was a significant increase in degenerated oocytes with 250 μM corticosterone (3.0 ± 1.4%, 2.4 ± 2.4%, and 22.4 ± 4.5% in control without DMSO, control with DMSO and 250 μM corticosterone, respectively; $P < 0.01$). The majority of degenerated oocytes were non-cleaved oocytes recorded after IVF and after 48 h in culture medium.

The progression of embryos to the blastocyst stage was monitored on day 5 of in vitro culture. Blastocyst development was assessed taking into account the number of blastocysts over the total number of embryos, with embryos being classified as described above.

On day 5 of culture, blastocyst development ($F_{5,30} = 22.7$; $P < 0.001$) differed between groups. Progression to the blastocyst stage did not differ between controls without DMSO and with DMSO ($P > 0.05$) and for oocytes that were exposed to up to 25 μM corticosterone during maturation (Fig. 1A). Hatching blastocysts developed in all groups pre-exposed to ⩽25 μM corticosterone and no differences between the proportions of each category of embryos were observed (Fig. 2A). The morphology of embryos that developed to the blastocyst stage in the various groups appeared normal when compared to control. On the other hand, when oocytes were pre-exposed to the highest concentration of corticosterone the ability of embryos to reach the blastocyst stage was completely hampered (Figs. 1A and 2A).

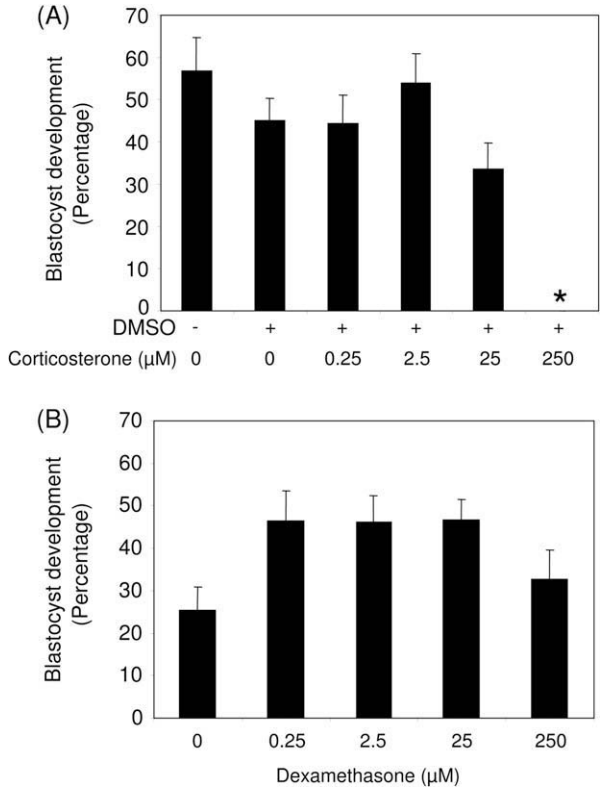

Fig. 1. Blastocyst development on day 5 of culture after mouse oocyte in vitro maturation, fertilization and embryo culture. Oocytes were matured in vitro in the presence of 0.25, 2.5, 25 or 250 μM corticosterone (A) or dexamethasone (B), and were then fertilized and cultured in vitro. Percentages of embryos that reached the blastocyst stage on day 5 of culture are shown. The data were analyzed using ANOVA and comparison between treatments was made using the *post hoc* Dunnett and Tukey tests (A) or were analyzed with the Mann–Whitney *U*-test (B). Asterisks denote significant differences ($P < 0.05$) when compared to the control groups: no corticosterone with DMSO (A) and no dexamethasone (B). This experiment was repeated on six occasions for corticosterone and on seven occasions for dexamethasone. Data are mean ± SEM.

3.2. Effects of dexamethasone on in vitro maturation, fertilization and culture of mouse oocytes

When oocytes were incubated in the presence of dexamethasone at increasing concentrations during IVM, no differences were found in the progression of oocytes to MII ($F_{4,30} = 0.6$; $P > 0.05$), and

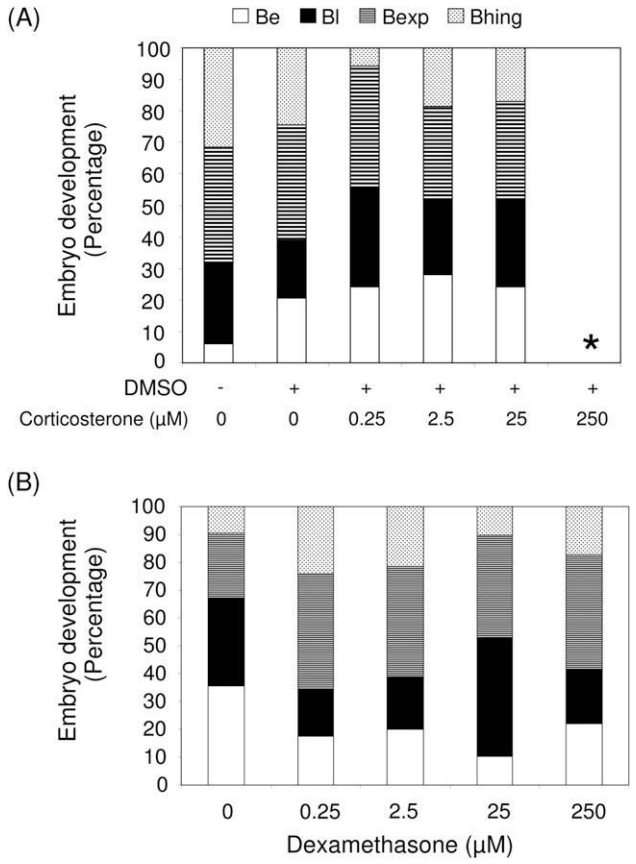

Fig. 2. Blastocyst development on day 5 of culture after mouse oocyte in vitro maturation in the presence of glucocorticoids, and subsequent fertilization and embryo culture. Oocytes were exposed to 0.25, 2.5, 25 or 250 μM corticosterone (A) or dexamethasone (B) during in vitro maturation. Blastocysts were classified as follows: Be, early blastocyst; Bl, blastocyst; Bexp, expanded blastocyst; Bhing, hatching blastocyst. Comparison between treatments was made using the Mann–Whitney *U*-test. Asterisks denote significant differences ($P < 0.05$) when compared to the control groups: no corticosterone with DMSO (A) and no dexamethasone (B). This experiment was repeated on six occasions for corticosterone and on seven occasions for dexamethasone.

the ability of mature oocytes to undergo fertilization was not affected ($F_{4,30} = 0.7$; $P > 0.05$) (Table 2). In addition, the ability of fertilized oocytes to undergo cleavage was not influenced by the presence of dexamethasone during IVM ($F_{4,30} = 0.8$; $P > 0.05$, cleaved over fertilized and $F_{4,30} = 0.5$; $P > 0.05$, cleaved over matured) (Table 2). In contrast to results obtained with corticosterone, dexamethasone did not increase the oocyte degeneration rate during maturation, fertilization and early cleavage (Kruskall Wallis ANOVA: 3.9; $P > 0.05$) (data not shown).

On day 5 of culture, there was no decrease in blastocyst development in the groups in which oocytes were pre-exposed to dexamethasone in comparison with values seen in the control group. (Fig. 1B). Embryos were able to progress up to hatching blastocysts at all concentrations of dexamethasone evaluated (Fig. 2B) and no differences were detected in gross morphology when compared to the control group. Thus, no inhibition was detected with any concentration of dexamethasone.

3.3. Phosphorylation patterns of ERK 1 and ERK 2 in mouse oocytes after IVM in the presence of corticosterone

Using a specific anti-phospho-MAPK antibody, no phosphorylated forms of MAPK were detected after immunoblotting in immature oocytes (Fig. 3A). In contrast, phosphorylated forms were detected after 17 h of maturation, showing there was activation of MAPK (control groups). Corticosterone was found to decrease MAPK activation after IVM in a concentration-dependent manner, as shown by the decrease of band intensity of p-ERK-1/2 (Fig. 3A) and densitometry (Fig. 3B). The activation of both ERK-1 and ERK-2 was considerably reduced by 250 μM corticosterone as shown by the major decrease of phosphorylated forms after Western blotting (Fig. 3A).

3.4. Phosphorylation patterns of ERK 1 and ERK 2 in mouse oocytes after IVM in the presence of dexamethasone

ERKs were in their inactive forms (dephosphorylated) in immature oocytes (Fig. 4A) whereas, in mature oocytes, phosphorylated forms of ERKs were detected in controls with no treatment. Little, if any, effect was observed on MAPK phosphorylation after IVM in the presence of dexamethasone, as compared to controls undergoing maturation without exposure to this glucocorticoid (Fig. 4A and B). The presence of dexamethasone did not inhibit ERK phosphorylation even at 250 μM (Fig. 4A and B), contrary to results with corticosterone which revealed a concentration-dependent inhibition.

4. Discussion

This study shows a differential effect of the natural glucocorticoid, corticosterone, and a synthetic one, dexamethasone, on mouse oocytes. Overall, a short exposure to high concentrations of corticosterone decreased in vitro oocyte maturation and, in mature oocytes pre-exposed to this glucocorticoid, it affected the

Table 2
Effect of exposure to increasing concentrations of dexamethasone during in vitro mouse oocyte maturation on progression to metaphase II and subsequent fertilization and embryo development.

Treatment	Maturation rate, % (No. MII/no. oocytes in culture)	Fertilization rate, % (No. fertilized/no. matured)	Cleavage, % (No. cleaved/no. fertilized oocytes)	Overall cleavage, % (No. cleaved/no. mature oocytes)
Control	87.3 ± 4.2 (112/128)	86.9 ± 3.9 (95/112)	87.6 ± 2.8 (84/95)	76.8 ± 2.8 (84/112)
Dexamethasone 0.25 μM	84.9 ± 3.6 (108/127)	81.6 ± 5.5 (87/108)	84.2 ± 5.3 (75/87)	68.6 ± 5.4 (75/108)
Dexamethasone 2.5 μM	88.9 ± 4.6 (114/129)	76.7 ± 3.6 (88/114)	93.5 ± 3.1 (82/88)	71.7 ± 4.2 (82/114)
Dexamethasone 25 μM	91.9 ± 3.0 (116/125)	78.1 ± 6.9 (89/116)	91.5 ± 4.0 (80/89)	70.4 ± 5.1 (80/116)
Dexamethasone 250 μM	87.3 ± 3.4 (107/124)	79.7 ± 5.0 (85/107)	89.1 ± 4.4 (75/85)	70.4 ± 4.1 (75/107)

Maturation rate is calculated as the number of oocytes in metaphase II (MII) over the total number of oocytes incubated. Fertilization rate is the number of oocytes fertilized over matured oocytes. Cleavage is expressed over fertilized oocytes and over matured oocytes. The data were analyzed using ANOVA. No differences were found on maturation and fertilization rates or cleavage ($P > 0.05$). Data (percentages) are mean ± SEM of seven independent experiments.

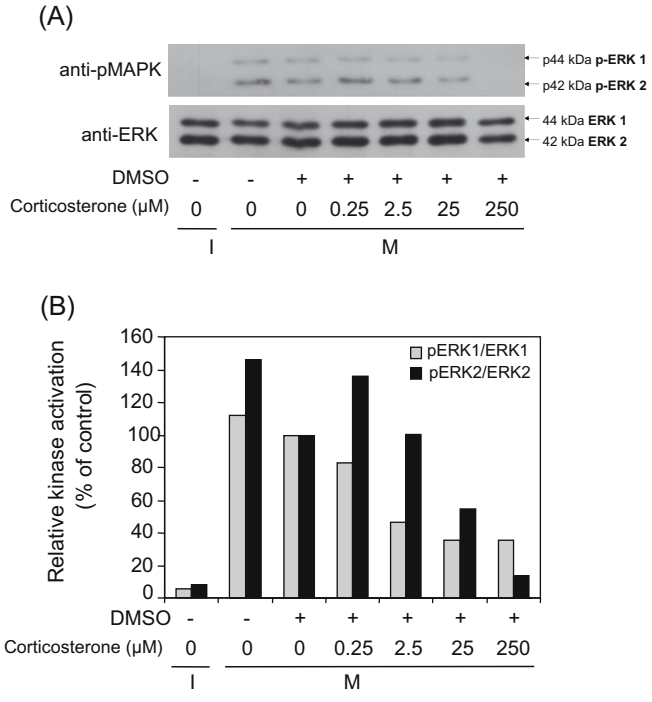

Fig. 3. Effect of corticosterone on ERK-1/ERK-2 activation after in vitro maturation of mouse oocytes in the absence or presence of corticosterone. (A) A representative immunoblot is shown following immunodetection of MAPK using specific antibodies for phosphorylated MAPK (pMAPK; top) and for total ERK (bottom). Lanes are: immature oocytes (I), mature oocytes (M) after 17 h of in vitro maturation in the presence of 0.25, 2.5, 25 or 250 μM corticosterone or its absence (0 μM; maturation control). An additional control group of maturation without corticosterone, but adding the solvent (DMSO) to culture media was included. (B) Densitometry of band intensities. ERK activation, as revealed by the level of phosphorylation, was measured and corrected by the total ERK at each treatment. The values of phosphorylated ERK-1/2 forms after 24 h of in vitro maturation (control solvent) were arbitrarily set to 100% and the other values were expressed relative to this level of activation.

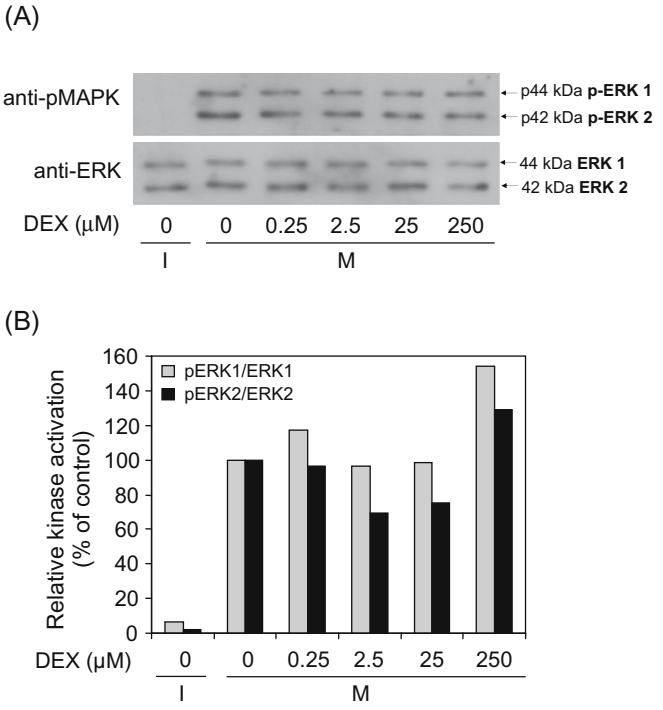

Fig. 4. Effect of dexamethasone (DEX) on ERK-1/ERK-2 activation after in vitro maturation of mouse oocytes in the absence or presence of dexamethasone. (A) A representative immunoblot is shown following immunodetection of MAPK using specific antibodies for phosphorylated MAPK (pMAPK; top) and for total ERK (bottom). Lanes are: immature oocytes (I), matured oocytes (M) after 17 h of in vitro maturation in presence of 0.25, 2.5, 25 or 250 μM dexamethasone or its absence (0 μM; maturation control). (B) Densitometry of band intensities is shown. ERK activation, as revealed by the level of phosphorylation, was measured and corrected by the total ERK at each treatment. The values of phosphorylated ERK-1/2 forms after 24 h of in vitro maturation (control) were arbitrarily set to 100% and the other values were expressed relative to this level of activation.

ability to undergo fertilization and early in vitro development up to the blastocyst stage. Thus, both nuclear and cytoplasmic maturation may be hampered. Corticosterone decreased phosphorylated forms of ERK-1/2 in exposed oocytes and this could represent an alteration in molecular mechanisms underlying oocyte maturation and competence for subsequent development. Dexamethasone, on the other hand, had no effect on in vitro oocyte maturation, fertilization or cleavage rate. Dexamethasone had little effect, if any, on ERK-1/2 phosphorylation.

Dexamethasone is a potent synthetic glucocorticoid with 25–50 times the glucocorticoid potency of cortisol, whereas corticosterone has a potency that is 80% that exhibited by cortisol (Magiakou and Chrousos, 1994). Dexamethasone has low affinity for binding proteins (Pugeat et al., 1981) and, thus, it has been used to explore the possible deleterious effects of the natural glucocorticoid on mouse oocytes. To our surprise, dexamethasone did not show the expected negative effect on oocyte maturation, fertilization, or subsequent development, and our results therefore suggest that the way the natural and synthetic glucocorticoids interact with glucocorticoid receptors on mouse oocytes may differ. Our results are in conflict with those by Van Merris et al. (2007), who found a negative effect of dexamethasone on oocyte maturation when used at 200 μM. It should be noted that a follicle bio-assay with a 12-day in vitro oocyte maturation was used in their study. Effects on meiosis progression could have been due to this long exposure to dexamethasone.

MAPKs are activated in response to several stimuli and they play pivotal roles controlling biological processes such as cell cycle arrest, cell proliferation and differentiation, or apoptosis (Pearson et al., 2001). The results obtained when oocytes were exposed to either corticosterone or dexamethasone during in vitro maturation, and the subsequent ability to undergo fertilization and culture were compared with results obtained examining MAPK activation at the end of in vitro maturation. Corticosterone was found to decrease MAPK activation (i.e. levels of phosphorylated forms) during in vitro maturation in a concentration-dependent manner. This inhibition was greatly noticeable at the highest concentration. Since substantial inhibition of progression through meiosis was only seen with 250 μM corticosterone, these results indicate that partial inhibition of ERK-1/2 by the presence of relatively high corticosterone concentrations (25 μM) during in vitro maturation allowed a high proportion of oocytes to progress to MII, and then undergo fertilization and cleavage up to the blastocyst stage. Only when ERK activation was considerably inhibited by corticosterone, oocyte maturation was significantly reduced with a rate that was half that seen in controls. Nevertheless, the effect was long-lasting because, of those oocytes that were able to mature, many had a reduced ability to undergo fertilization and none was able to develop to the blastocyst stage. Dexamethasone, on the other hand, had no effect whatsoever on MAPK activation during in vitro maturation and this glucocorticoid did not have any effect on progression through meiosis either. Furthermore, oocytes that matured in the presence of dexamethasone were not affected in their ability to undergo fertilization or embryo development. Thus, in general terms, there was a good agreement between either a negative effect or no effect on MAPK activation and similar effects on oocyte progression through meiosis.

Little is known about the underlying mechanisms of glucocorticoid action during in vitro maturation. The inhibitory effect of cortisol and dexamethasone on pig oocyte meiosis resumption and maturation (Yang et al., 1999) was partially attributed to a reduced amount of p34cdc2-cyclin B1 complex (MPF) (Chen et al., 2000). The activation of MPF occurs before germinal vesicle breakdown (GVBD) and its inhibition by various means prevents GVBD in mammals (Kubelka et al., 2000; Mermillod et al., 2000) including the mouse (Choi et al., 1991). It is possible that the prolonged interval until meiosis resumption that occurs in the pig (about 24 h) allowed glucocorticoids to block MPF kinase activation, and thus the progression into M phase. Since the activity of MPF was not measured in the present study, we cannot speculate about the possible effects of glucocorticoids on this kinase in mouse oocytes.

Cumulus expansion was less apparent after maturation of oocytes exposed to 250 μM corticosterone. This could reflect a reduced ability of these oocytes to mature in vitro, in comparison to other treatments. Recent studies supplied evidence that activation of MAPK (ERK-1/2) in cumulus cells mediates the LH-stimulated resumption of meiosis, involving down-regulation of communication between granulosa cells and, possibly, connexin-43 phosphorylation. This suggests a link between MAPK and cumulus expansion (Liang et al., 2007; Sela-Abramovich et al., 2005; Su et al., 2003) and opens up the possibility that the effect of glucocorticoids may be mediated, at least in part, via an effect on cumulus cells. A possible interaction of glucocorticoids with ERK-1/2 in cumulus cells has not been explored yet and deserves future attention.

Previous in vitro studies have assessed the effect of glucocorticoids on mammalian oocytes and embryos using various endpoints: the ability to undergo oocyte maturation in the mouse (Andersen, 2003), oocyte maturation and the subsequent ability of exposed oocytes to undergo fertilization in the pig (Yang et al., 1999), or in vitro follicle differentiation and oocyte maturation, together with early embryonic development in the mouse (Van Merris et al., 2007). However, it is important to note that, in studies using the mouse, the natural endogenous glucocorticoid corticosterone was not evaluated in any of them (Andersen, 2003; Van Merris et al., 2007) with cortisol being used instead. The differences between studies of glucocorticoid effects on oocyte maturation could be ascribed to differences in methodology or to species differences, mainly at glucocorticoid receptor level and ligand-affinity for the receptors, but also on protein kinetics during oocyte maturation.

In conclusion, the present study showed a differential effect of synthetic and natural glucocorticoids on mouse oocytes in vitro as reflected by the different effects on embryo development after glucocorticoid exposure during in vitro maturation of oocytes. Only high levels of corticosterone altered the progression through different stages up to the blastocyst stage. The decreased activation of ERK-1/2 during maturation in the presence of corticosterone could lead to a reduced subsequent ability to generate embryos, but it remains to be determined how the native glucocorticoid causes a decrease in ERK phosphorylation. Although extrapolation to an in vivo situation should be made with caution, it is possible to conclude that the exposure of oocytes to high levels of glucocorticoids may alter the normal molecular mechanisms of oocyte maturation, having negative consequences for subsequent embryo production. However, the concentration of corticosterone at which an effective inhibitory effect was detected in vitro is far above the levels reached in vivo (Shanks et al., 1990). Thus, it is possible that a short exposure of the female gamete to elevated concentrations of glucocorticoids during stressful situations (or during a short exposure to glucocorticoid therapy) may not represent a major hazard to meiosis progression of the oocyte.

Conflict of interest statement

There are no conflicts of interest.

Acknowledgements

We thank Anthony Michael for suggestions and many helpful discussions. We are grateful to the staff of the Servicio de Imagen Científica, Instituto de Investigaciones Biomédicas (CSIC), Madrid, Spain, for allowing us to use its facilities. Supported by the Spanish Ministry of Education and Science (Grants REN 2003-01587, CGL2006-13340/BOS and CGL2005-02354/BOS). RG received a studentship from the I3P-CSIC programme. ERSR is the recipient of a Royal Society Wolfson Research Merit Award.

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