The effect of glucocorticoids on ERK-1/2 phosphorylation during maturation of lamb oocytes and their subsequent fertilization and cleavage ability in vitro

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High levels of glucocorticoids may alter reproduction, but little is known about their direct actions on oocyte maturation, fertilization and subsequent development. Earlier work suggested negative effects of cortisol or dexamethasone on oocyte maturation but differences were noted between animal models. Both glucocorticoids reduce the p34cdc2–cyclin B1 complex but it is unknown if other signaling pathways important for meiosis progression are affected. In this study, using sheep oocytes as a model system, we assessed in vitro the effects of increasing concentration of glucocorticoids (0–250 μM) on oocyte maturation and underlying changes in the MAP kinase pathway, and the ability of oocytes to undergo fertilization and embryo development. Cortisol decreased oocyte maturation but only at the highest concentration, whereas dexamethasone had no effect. Fertilization and cleavage were not affected. On the other hand, both cortisol and dexamethasone inhibited ERK-1/2 activation in a concentration-dependent manner. It thus seems that oocytes can overcome deleterious effects of glucocorticoids during maturation despite the decrease in ERK-1/2 activity, but repercussions in vivo should be further explored.

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1. Introduction

The physiological response to stress includes the release of catecholamines and glucocorticoids which act alleviating its effects. Glucocorticoids can influence reproduction acting on the hypothalamus–pituitary axis or on the gonads and it is accepted that chronic stress inhibits reproduction [1]. Moreover, high amounts of glucocorticoids administered during medical therapy or produced in Cushing’s syndrome or other diseases [2] could also have negative consequences on reproduction. There is evidence suggesting disruptive effects of stress on assisted reproductive techniques in wild animals [3,4] and stress is considered to be a contributing factor of reproductive failure in wild species maintained in captivity [5].

We have previously reported a possible effect of stress on the limited success of assisted reproductive procedures in a critically endangered ungulate [6]. A rise in cortisol levels during the days of more intense management has been observed during the superovulation treatment of gazelles for recovering immature oocytes, whereas cortisol levels were lower in animals tranquilized with a long acting neuroleptic [7]. Oocytes obtained from the latter group progressed to metaphase II at a higher rate than untreated controls, suggesting a possible negative effect of glucocorticoids on in vitro oocyte maturation [7].

Studies concerning the direct effect of glucocorticoids on oogenesis have shown conflicting results. In vitro maturation (IVM) of pig oocytes was found to be inhibited in a concentration- and time-dependent manner by glucocorticoids (cortisol and dexamethasone) when added to culture media [8]. In addition, a follicle bioassay showed the toxic potential of dexamethasone on mouse ovarian function and early embryo development [9]. However, cortisol and dexamethasone did not affect meiosis resumption and maturation rate of mouse oocytes undergoing FSH-induced and spontaneous IVM [10]. In women, oocytes that failed at fertilization came from follicles with higher cortisol levels in comparison to those that yielded oocytes that were able to fertilize and cleave [11]. On the other hand, a possible role of glucocorticoids in oocyte maturation and ovulation has also been suggested [12]. These discrepancies could be due, in part, to differences between species and strongly suggest that a single animal model may not be suitable to understand drug/chemical toxic actions on oocyte maturation and fertilization and their underlying molecular mechanisms. For example, clear differences exist in the timing of oocyte maturation, or mechanisms underlying resumption of meiosis, between rodents and ungulates.
To the best of our knowledge, no studies have focused on the direct effect of glucocorticoids on ruminant oocytes. Sheep oocytes were therefore chosen as a model system for our studies. Sheep oocytes may be regarded as a good model system to address these questions because they are readily available in large quantities and earlier work has characterized conditions for in vitro maturation, fertilization and early development. Oocytes from this species may also serve as a model for the assessment of the direct effects of stress on oocyte maturation that may occur in endangered ungulates because the scarcity and value of material that can be obtained from these endangered species preclude their use in this type of studies.

Glucocorticoids are steroid hormones released from the adrenal cortex. Their action is mediated through the nuclear glucocorticoid receptor, promoting the synthesis of mRNA in the nucleus of target cells and leading to the synthesis of specific proteins responsible of the cellular response. This mechanism of action is the cause of the multiorganic effects of glucocorticoids, although nongenomic actions of glucocorticoids are also known [13,14]. Cortisol is the most potent of the natural glucocorticoids. It is inactivated to cortisone in mineralocorticoid target tissues. The inverse conversion from cortisone to cortisol takes place in glucocorticoid target tissues. This cortisol/cortisone shuttle is regulated by isoenzymes of 11-hydroxysteroid dehydrogenase (11βHSD), HSD1 and HSD2 [15]. Dexamethasone is a synthetic steroid with similar structure to cortisol, but it has 25–50 times the glucocorticoid potency of cortisol [16] and it is also a substrate for 11βHSD both in vitro and in vivo [17]. Glucocorticoids diffuse from blood to the follicular fluid [18] and the local concentration of cortisol increase in the preovulatory follicle [12,19] regulated by 11βHSD. Follicular metabolism of glucocorticoids has been linked to assisted conception. An increased probability of conception in women undergoing in vitro fertilization-embryo transfer was associated with high follicular cortisol:cortisone ratios [20]. On the contrary, 11βHSD enzymes predominantly inactivate cortisol in pig oocytes, and this mechanism may be important to avoid the deleterious action of glucocorticoids on the maturation of oocytes in this species [21].

Little is known about the underlying mechanisms of glucocorticoid action on oocytes during in vitro maturation. The inhibitory action of cortisol and dexamethasone in porcine oocytes [8] was partially attributed to the reduction in p34cyc2-cyclin B1 complex (maturation/metaphase-promoting factor, MPF) [22], but there are no studies assessing a possible effect on other signaling pathways. The MPF complex together with the mitogen-activated protein kinases (MAPKs) play pivotal roles in regulating the maturation of the oocyte by an intricate and coordinated interplay including cross-talk with other components and cell signals [23,24]. It is therefore possible that, in addition to MPF, glucocorticoids may affect MAPK activity. MAPKs are serine/threonine kinases that become activated by phosphorylation on threonine and tyrosine residues [25]. The extracellular signal-regulated kinases (ERKs), ERK-1 and ERK-2 are members of the MAPK family activated during oocyte maturation [26].

We have designed experiments to test the hypothesis that glucocorticoids (cortisol and dexamethasone) may negatively affect oocyte maturation and the underlying MAPK activation. In testing such hypothesis, dexamethasone was used as positive control, to seek an enhancement of effects upon oocytes, and cortisol was employed as a negative control, since it is the inactive metabolite of cortisol. If an effect by cortisol were observed, we hypothesized that it could be due to a conversion to cortisol by 11βHSD. Epidermal growth factor (EGF) plays an important role in cell proliferation, growth and differentiation [27] and induces ERK short-lived activation [28]. This growth factor is often included in culture media due to its positive role promoting in vitro maturation or inducing meiosis resumption. We hypothesized that inclusion of this com-pound could help the oocyte to overcome the possible inhibitory effect of glucocorticoids on the oocyte, and thus the absence of EGF in culture media was also examined. Finally, we tested if exposure to glucocorticoids during oocyte maturation would impact on fertilization or subsequent development.

The aims of the present study were thus to (1) assess the effects of the natural (cortisol) and the synthetic glucocorticoid (dexamethasone) on in vitro oocyte maturation, and the subsequent ability to undergo fertilization, using sheep oocytes as model system, and (2) evaluate whether cortisol and dexamethasone exert an effect on phosphorylation patterns of ERK-1/2 during in vitro oocyte maturation in this model.

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. Collection of oocytes and in vitro maturation

Ovaries from prepubertal lambs were collected at a local abattoir and transported to the laboratory within 1 h at 25–30 °C in 0.9% (w/v) NaCl solution supplemented with penicillin and streptomycin (50 μg/ml each). Cumulus oocyte complexes (COCs) were recovered by slicing and mincing the ovaries with the aid of 18-gauge needles fixed to disposable syringes in Hepes-TCM-199. Collecting medium consisted of TCM-199 (M-5017) containing 25 mM Hepes, 4.76 mM sodium bicarbonate, 2.5 mM glutamine, 0.36 mM sodium pyruvate, 0.1% (w/v) polyvinyl alco-hol (PVA) and 50 μg/ml gentamicin (Gibco, Madrid, Spain). Only COCs with a uniform ooplasm and a compact cumulus cell mass were selected for in vitro culture and randomly assigned to each treatment. After rinsing 3–4 times in Hepes-buffered TCM-199 and once in IVM medium, the COCs were placed in 4-well culture dishes with 500 μl of IVM medium per well. Cortisol (hydrocortisone, H-0888), dexamethasone (D-1159) or cortisone (C-2755) were added as positive control, to seek an enhancement of effects upon oocytes, and 10 μg ovine FSH/LH/ml, 1 mg estradiol/ml, 10 ng EGF/ml [29], 200 μM cysteamine [30], 0.36 mM sodium pyruvate and 2 mM glutamine. Mineral oil was not used to over-lay media to avoid possible absorption of steroids [31,32]. Oocytes were cultured at 38.5 °C under 5% CO2 in air and maximum humidity for 24 h. After the experiments, groups of approximately 20–30 COCs were cultured in 4-well plates with 500 μl of IVM medium per well. Cortisol (hydrocortisone, H-0888), dexamethasone (D-1159) or cortisone (C-2755) were added at the beginning of culture as indicated in Section 2.8.

2.3. In vitro fertilization

At the end of IVM, oocytes, presumed mature, were washed in Hepes-buffered TCM-199 followed by gentle pipetting with the aid of a automatic micropipette to dissociate the surrounding cumulus cells, but leaving corona cells intact. Fertilization medium was synthetic oviduct fluid (SOF) [31] supplemented with inactivated 2% (v/v) FCS, 1 μg hypotaurine/ml and 10 μg heparin/ml. Oocytes were co-incubated with frozen-thawed spermatozoa collected after swim-up. Sperm from adult rams was cryopreserved in 0.25 ml straws in Fiser’s extender. Straws were thawed for 30 s at 37 °C in a water bath. Spermatozoa in 100 μl of the cryodiluent was poured into a conical centrifuge tube and overlaid with 500 μl of Hepes-buffered SOF medium and incubated at 38.5 °C in air for 30 min, followed by sperm recovery from the top of the sperm suspension. The final concentration of spermatozoa used during gamete co-incubation was 3 × 105 motile spermatozoa/ml. Gametes were incubated in 4-well culture dishes without mineral oil for 18–20 h at 38.5 °C under 5% CO2, in air.

2.4. In vitro culture

After gamete co-incubation, presumptive zygotes were washed in culture medium to remove spermatozoa and cell debris. They were transferred to 500 μl of SOF medium supplemented with 0.4% (w/v) bovine serum albumin (BSA), 1 mM glutamine, 1% (v/v) non-essential and 2% (v/v) essential amino acids (Gibco) and cultured at 38.5 °C under a 5% CO2 in air with maximum humidity. At 42–44 h after insemination, non-cleaved (one-cell) oocytes were separated from those that cleaved and stained (see below). Cleaving oocytes had medium renewed after 72 h of culture and also on day 5 when 10% (v/v) FCS was added instead of BSA. Day of fertilization was considered day 0.

2.5. Evaluation of meiosis progression

The resumption and progression through meiosis was evaluated after 24 h of in vitro maturation or retrospectively after in vitro fertilization and culture. Non-cleaved oocytes were stained with Hoechst 33342 (10 μg/ml) and visualized in a microscope equipped with epifluorescence to determine maturation status and/or

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fertilization by the presence of ≥2 pronuclei or decondensed sperm heads. Parthenogenic activation was also examined after maturation and it was calculated as the number of activated oocytes over the number of matured oocytes after 24 h of incubation. Oocytes denuded of a pronucleus and one polar body, or two pronuclei without polar bodies, were considered to be parthenogenetically activated. Some oocytes were also found in anaphase or telophase II stage, and they were also regarded as activated. 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 DNA400 chromatic beam splitter and a 420 barrier filter.

2.6. SDS-PAGE and immunoblotting

For the detection of phospho-ERK (p-ERK) and ERK proteins, 30 denuded oocytes per treatment were collected, washed 5 times in phosphate-buffered saline (PBS) with 0.1% (w/v) polyvinylpyrrolidone (PVP) and transferred in 5 μL of PBS plus PVP to centrifuge tubes. Samples were immediately frozen and stored at −80 °C until assays were performed. Oocytes were lysed in 5 μL of double-strength sodium dodecyl sulfate (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 into two aliquots and each was separated in a 9% SDS gel with a 100:1 acrylamide:bisacrylamide ratio. Each gel was transferred to Immobilon-P membranes (Millipore, Bedford, MA). Both blots were blocked for 1 h in 5% (w/v) non-fat dry milk in Tris-buffered saline pH 7.4 with 0.05% Tween-20 (TTBS). One blot was incubated overnight in 5% (w/v) BSA TTBS with anti-phospho-p44/42 MAP kinase rabbit antibody (#9101, dilution 1:1000; Cell Signalling, Barcelona, Spain) at 4 °C. The other blot was incubated for 1 h in 5% (w/v) milk solution-TTBS with anti-total-ERK rabbit polyclonal antibody (sc-9948, dilution 1:1000; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Blots were then incubated with each antibody and were then developed with enhanced chemiluminescence (ECL). Western blotting detection reagent (RP2108, Amersham, Madrid, Spain). Band intensities were quantified by densitometry using the public domain digital imaging and analysis NIH-image J software (http://rsb.info.nih.gov/ij/). The level of ERK activation of each treatment point was measured and corrected by the total ERK (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 activity.

2.7. Statistics

The data were analyzed using ANOVA and when significant differences were found, comparison between treatments was made using the post hoc Dunnett or Tukey tests. Variables were expressed as percentages and they were arcsine-transformed to fulfill parametric assumptions. Results are presented as mean ± S.E.M. 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).

2.8. Experimental design

Dexamethasone is a synthetic and potent steroid with a structure similar to that of cortisol but with higher potency [16] and it was used to enhance the potential effects of glucocorticoids on oocytes. Cortisone, the inactive metabolite of cortisol, was expected to have no effects and was used as a negative control. Cortisol (hydrocortisone, H-0888) was dissolved in DMSO (D-2650). Cortisone (C-2755) and dexamethasone (D-1159) were diluted in milli-Q grade water. Stock solutions of various concentrations (0.05, 0.5, 5 and 50 mM) 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 group without cortisol, but with the same volume of DMSO was included as control.

2.8.1. Effect of glucocorticoids on in vitro maturation of lamb oocytes

In a first series of experiments, cortisol, cortisone and dexamethasone were added to incubation media at 0.25 μM (~100 ng/ml) final concentrations. We used these glucocorticoid concentrations because they are similar to the cortisol concentrations found in blood plasma under stressful conditions in sheep [34] and in gazelles during the days of more intensive handling of assisted reproductive procedures [7]. An additional group with cortisol, but without EGF was used, to test a possible interaction between EGF and cortisol. A group of oocytes without any cortisol but with higher potency [16] and it was used to enhance the potential effects of glucocorticoids on oocytes. Cortisone, the inactive metabolite of cortisol but with higher potency [16] and it was used to enhance the potential effects of glucocorticoids on oocytes. Some oocytes were also found in anaphase or telophase II stage, and they were also regarded as activated. 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 DNA400 chromatic beam splitter and a 420 barrier filter.

2.8.2. Effects of cortisol and dexamethasone on in vitro maturation, fertilization and culture of lamb oocytes

Cortisol or dexamethasone were added at increasing concentrations (0, 0.25, 2.5, 25 and 250 μM) to incubation media to determine whether an effect is observed at elevated glucocorticoid concentrations. These concentrations were similar to those used in earlier in vitro studies and were employed to allow comparisons with previous work [8–10]. An additional group with solvent alone (DMSO) was used. Oocytes were incubated for 24 h and then stained to evaluate nuclear maturation or co-incubated with spermatozoa to assess the ability of the oocytes to undergo fertilization and develop in culture. Experiments were repeated on separate occasions. For cortisol, they were carried out seven times for IVM and three times for in vitro fertilization. For dexamethasone, experiments were repeated eight times for IVM and on three occasions for in vitro fertilization.

2.8.3. Phosphorylation patterns of ERK-1 and ERK-2 in lamb oocytes after IVM in the presence of cortisol or dexamethasone

Cortisol or dexamethasone were added at increasing concentrations to incubation media and oocytes incubated for 24 h as described above. After culture, oocytes were denuded, proteins extracted, separated and quantified after Western blotting and immunostaining as described above. A group of immature oocytes was also included as controls. Two independent experiments were carried out for each steroid.

3. Results

3.1. Effect of glucocorticoids on in vitro maturation of lamb oocytes

A total of 570 oocytes was used to test a possible effect of cortisol on cortisol in vitro maturation of lamb oocytes, using dexamethasone and cortisone as a positive and negative controls, respectively. No significant differences were observed in the proportion of oocytes undergoing nuclear maturation between control and groups with cortisol, dexamethasone or cortisone added at 0.25 μM (~100 ng/ml) (F5,10 = 0.4; p > 0.05) (Table 1). There was no effect of cortisol regardless of whether it was included in the presence or the absence of EGF during IVM (p > 0.05). In addition, there was no effect of DMSO, the solvent used for cortisol (p > 0.05) (Table 1).

No differences were found in parthenogenetic activation of oocytes (F5,10 = 1.0; p > 0.05) when the various glucocorticoids were added to culture media at 0.25 μM (~100 ng/ml) in comparison to the control group (Table 1).

3.2. Assessment of increasing concentrations of cortisol on oocyte in vitro maturation, fertilization and culture

A total of 900 oocytes was employed to evaluate the effect of cortisol at increasing concentrations on in vitro maturation. The presence of DMSO, the solvent used for cortisol, did not have any effect on IVM compared to control (p > 0.05) (Table 2). Cortisol added at 0.25, 2.5 or 25 μM did not differ from controls (groups with or without DMSO) (p > 0.05). However, significant differences (p < 0.05) in maturation rates were found when cortisol was added at 250 μM with the steroid exerting a clear inhibitory effect (Table 2).

After incubation under in vitro maturation conditions, 313 oocytes without or with prior exposure to cortisol were co-incubated with spermatozoa. Fertilization and subsequent cleavage were not statistically different (p > 0.05) between controls and cortisol-treated oocytes at all the concentrations examined (0.25–250 μM) (Table 2).

During IVM, the inclusion of cortisol resulted in no significant differences in the proportion of activated oocytes between treated oocytes and control oocytes exposed to DMSO alone (p > 0.05) (data not shown).

3.3. Assessment of increasing concentrations of dexamethasone on oocyte in vitro maturation, fertilization and culture

A total of 1068 oocytes was used to test the effect of different concentrations of dexamethasone on in vitro maturation. No significant differences were found on maturation rates between any of the concentrations tested and control (F4,35 = 0.5; p > 0.05).
After IVM, 384 oocytes without or with prior exposure to dexamethasone were co-incubated with spermatozoa. Fertilization and cleavage rates did not differ between treatments or between them and control (p > 0.05) (Table 3).

There was an increase in parthenogenetic activation when oocytes were incubated with dexamethasone (Fig. 1) with values of about 30% parthenogenesis with the highest dexamethasone concentration. An ANOVA revealed significant differences (F₂,₉₀ = 3.9; p < 0.05) between treatments. Post hoc analysis showed that parthenogenesis was significantly higher in the group of oocytes exposed to 250 μM dexamethasone in comparison to the control group (p < 0.05) (Fig. 1). No significant differences were detected in the other groups in relation to the control.

3.4. Phosphorylation patterns of ERK-1 and ERK-2 in lamb oocytes during IVM in the presence of cortisol

After resolving oocyte proteins by SDS-PAGE and immunodetection using a specific anti-phospho-MAPK antibody, no ERK-1/2 activation was detected in immature oocytes, i.e., ERKs were in their inactive form (dephosphorylated) (Fig. 2(A)) in agreement with previous studies in other species [23,24]. After oocyte IVM, ERK-1/2 activation was evident with slight differences noted in activation levels depending on whether DMSO was absent or present during culture (Fig. 2(A) and (B)). Cortisol was found to reduce, in a concentration-dependent manner, both ERK-1 and ERK-2 phosphorylation levels when added to culture media during IVM of lamb oocytes (Fig. 2(A)). To quantify the degree of inhibition, the level of MAPK activation was arbitrarily set to 100% in the control group containing DMSO (solvent control). Results showed a statistically significant difference between control (with DMSO) and cortisol at 2.5 μM or higher concentrations in both ERK-1 and ERK-2 (Fig. 2(B)). Kinase activation was inhibited up to 60% of control levels with the highest concentration tested (Fig. 2(B)). The decrease in phosphorylated forms related to controls appeared to be higher for ERK-1 than for ERK-2 (Fig. 2(B)).

3.5. Phosphorylation patterns of ERK-1 and ERK-2 in lamb oocytes during IVM in the presence of dexamethasone

As in the preceding experiment, no phosphorylated forms of MAPK were seen in immature oocytes (Fig. 3(A)). After oocyte maturation, levels of kinase activation were high (Fig. 3(A) and (B)). A decrease in phosphorylated forms of ERK-1 and ERK-2 were observed after IVM of oocytes in the presence of dexamethasone when compared to the control group (Fig. 3(A)). This inhibition in MAPK activation was concentration-dependent, reaching about half of the maximum activation level seen with the highest concentration of dexamethasone (250 μM) (Fig. 3(B)).

4. Discussion

This study, using sheep oocytes as model system, shows for the first time an inhibitory effect of the glucocorticoids cortisol and dexamethasone on MAPK activation during in vitro oocyte maturation. Furthermore, this study shows an inconsistent effect of these glucocorticoids on oocyte progression through meiosis, with cortisol inhibiting and dexamethasone having no effect. None of these glucocorticoids affected the ability of oocytes that matured...
in their presence to undergo fertilization and early development. Our results on oocyte in vitro maturation are at variance with results in some earlier studies using other model species (i.e., pig, mouse) that have different timing and mechanisms of oocyte maturation, thus highlighting that caution should be used when extrapolating results between mammalian models. The results of our study also suggest that sheep oocytes may be an adequate model to provide insight into underlying pathways of drug/chemical reproductive or developmental toxicity for domestic and wild ungulates.

Cortisol was tested using a wide range of concentrations. The range of cortisol concentrations tested in this study included both physiological and supraphysiological levels of this glucocorticoid [2,19] and these concentrations are similar to the glucocorticoid concentrations used in previous studies [8–10]. The highest concentration of cortisol (250 µM) was able to inhibit meiosis progression to metaphase II although oocytes that did mature showed no alteration in fertilization and cleavage rates. This result seems to suggest that cytoplasmic maturation may not be completely affected by the presence of cortisol. The ability of embryos to develop up to the blastocyst stage could not be evaluated and this should be examined in more detail in the future. In studies using mouse oocytes, cortisol (0.1–10 µg/ml; ~0.25–25 µM) had no effect on germinal vesicle breakdown (GVBD) rates or extrusion of polar bodies during in vitro maturation [10]. On the other hand, work in vitro on pig oocytes has revealed an inhibitory effect of cortisol on GVBD rates with a minimal effective dose of 0.1 µg/ml (0.25 µM) [8].

Dexamethasone, a potent synthetic glucocorticoid, was used to enhance a potential deleterious effect of glucocorticoids on oocytes. However, we did not observe any significant difference in relation to controls in maturation, fertilization or cleavage rates when dexamethasone at up to 250 µM (~100 µg/ml) was added to culture media. Our results of in vitro culture of sheep oocytes are in agreement with the results reported for mouse oocytes for which no effect was found in GVBD rates or polar body extrusion after IVM in the presence of dexamethasone (1–20 µg/ml; ~2.5–50 µM) [10]. On the other hand, Van Merris et al. [9] found that although up
Fig. 3. Effect of dexamethasone (DEX) on ERK-1/ERK-2 activation after in vitro maturation (IVM) of lamb oocytes. A representative immunoblot (A) 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 24 h of IVM in the presence of 0.25, 2.5, 25 or 250 μM of dexamethasone or absence of the glucocorticoid (0 μM; maturation control). Kinase activation after each treatment, as revealed by level of phosphorylation, was measured and corrected by the total ERK (B). Values of phosphorylated ERK-1/2 forms after 24 h of IVM (controls) were arbitrarily set to 100% and the other values were expressed relative to this level of activation. Densitometry of band intensities was performed using the public domain digital image processing and analysis NIH-Image J software (http://rsb.info.nih.gov/ij/). The results shown are representative of two experiments. Asterisks denote significant differences (p < 0.05) in comparison with the control group (i.e., 0 μM dexamethasone; maturation control).

to 40 μg/ml (~100 μM) dexamethasone had no effect on folliculogenesis and oogenesis in a mouse follicle bioassay, concentrations of 80 μg/ml (~200 μM) dexamethasone disturbed folliculogenesis and resumption of meiosis and decreased maturation rates; development to the blastocyst stage was also impaired [9]. Studies in vitro using pig oocytes have noticed an inhibitory effect of dexamethasone on oocyte maturation around the time of GVBD and their activities remain at high levels until metaphase II, preventing parthenogenetic activation; after fertilization or parthenogenetic activation, MAPK becomes dephosphorylated resulting in loss of activity [35]. MAPK inactivation is required for normal spindle function and polar body emission [36] and it is associated with pronuclear formation [37]. The decrease in phosphorylated active forms of ERK-1 and ERK-2 during oocyte incubation in the presence of glucocorticoids could explain, in part, the increase in parthenogenetic activation found in this study, although this would only be the case for dexamethasone. Another possibility could be that glucocorticoids are somehow causing oocyte activation by other means and this is eventually leading to MAPK inactivation. We cannot distinguish, based on our present results, whether parthenogenetic activation is the consequence or the cause of MAPK inactivation. Although there was a concentration-dependent decrease in ERK-1/2 active forms after oocyte incubation in the presence of cortisol (with up to 50% inhibition of MAPK activation), parthenogenetic activation was not triggered by this glucocorticoid. On the other hand, it is known that MAPK activity decreases several hours after fertilization or parthenogenetic activation [23,35]. Thus, if dexamethasone is causing activation of mature oocytes (and an ensuing MAPK inactivation), it would mean that lamb oocytes completed their in vitro maturation hours before the end of the 24-h culture in order to respond to dexamethasone and form a pronucleus. This latter scenario appears unlikely.

It is unknown by which mechanisms glucocorticoids could mediate, directly or indirectly, their inhibitory effects on MAPK activation in sheep oocytes. A possible explanation may relate to their interaction with the Mos-MEK-MAPK pathway. No evidence has been presented showing the expression of Mos, the product of the c-mos proto-oncogene, in ovine oocytes, but expression of Mos has been identified in cow oocytes, a related species [38]. Evidence from other species suggests that, in oocytes, Mos is an upstream activator of ERK-1/2. In Xenopus oocytes, Mos activates MEK, a specific regulator of ERK [24]. In mice, the exposure of mature eggs to the MEK inhibitor U0126 resulted in parthenogenetic activation, yielding the same phenotypes as Mos−/− parthenogenotes [36]. Mos−/− oocytes activate spontaneously exhibiting specific phenotypes attributed to the absence of MEK and MAPK activities in MII oocytes. The inhibition of MAPK also resulted in parthenogenetic activation in rat oocytes [39] and pig oocytes [40]. Future studies should examine the inhibition of the MAPK pathway in sheep oocytes to assess a possible relation between MAPK inhibition and parthenogenes. The use of glucocorticoid receptor antago-
nists could help to elucidate if the effects observed in the present study are mediated through glucocorticoid receptors on oocytes. The effects of glucocorticoids on the activation of MAPK pathways may in fact be very complex, as revealed by studies on somatic cells. Previous studies using cell lines have shown rapid effects of glucocorticoids on the phosphorylation patterns of distinct members of the MAPK family. An inhibitory effect of dexamethasone in the A-459 human adenocarcinoma cell line appeared to be specific for the Raf/MEK-1/ERK-1 pathway with ERK-2 being unaffected [13]. In a different study, corticosterone was found to induce a rapid activation of MAPK 14 (formerly known as p38) and MAPK 8/9 (formerly known as JNK) [14] and also ERK-1/2 [41] in PC12 cells.

In summary, we have carried out studies examining the effects of glucocorticoids on in vitro maturation (and underlying molecular mechanisms such as MAPK activation), fertilization and early embryo development using sheep oocytes as model system. Our results revealed a reduced percentage of in vitro matured sheep oocytes in the presence of high concentrations of cortisol, but not of dexamethasone, suggesting that physiological or even controlled therapeutic levels of glucocorticoids could not represent a hazard to the maturation of the oocyte. On the other hand, a decrease in ERK activation after IVM was found with both compounds. The inhibition of ERK observed after IVM was not complete and this may have allowed oocytes to progress through the cell cycle even with reduced levels of ERK activation, although altered ERK-1/2 signaling may lead to various pathological effects [28]. Thus, the pathways by which glucocorticoids could mediate their effects on MAPK phosphorylation during oocyte in vitro maturation deserve future attention because this may have potential deleterious effects on oocyte maturation and oocyte competence for post-fertilization development. Actions to reduce the effects of glucocorticoids during assisted reproduction may be important to improve embryo production and should be explored further.

Conflict of interest statement

None.

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