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USE OF RETINOIDS DURING OOCYTE MATURATION DIMINISHES APOPTOSIS IN CAPRINE EMBRYOS

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Exposure of caprine oocytes and embryos to retinoids enhances embryonic development, but the mechanisms governing this phenomenon have not been characterised. The aim of the present study was to evaluate if the incidence of apoptosis is affected by the addition of retinyl acetate (RAc) and 9-cis-retinoic acid (RA) during *in vitro* maturation (IVM) of caprine oocytes. Embryonic development was recorded on days 3 and 8 post-fertilisation, and apoptosis was measured by caspase activity and DNA fragmentation (TUNEL assay). Control zygotes had lower capacity to cleave and reach the blastocyst stage (24.45 ± 2.32 and 5.32 ± 0.81, respectively) than those of RAc- (29.96 ± 1.62 and 7.94 ± 0.93, respectively) and RA-treated groups (30.12 ± 1.51 and 7.36 ± 1.02, respectively). Oocytes and blastocysts positive for TUNEL assay were more frequent, respectively, in the controls (8.20 ± 0.78, 8.70 ± 1.05) than in RAc (5.60 ± 0.52, 4.80 ± 0.51) and RA (6.40 ± 0.69, 5.40 ± 0.69). Caspase activity did not differ between control oocytes (7.20 ± 0.91), RAc (6.60 ± 0.68) and RA (7.30 ± 0.67), but it was reduced in RAc- (5.05 ± 0.62) and RA-treated blastocysts (5.75 ± 0.22) compared to controls (8.35 ± 0.71). These results indicate that the addition of retinoids during IVM increases the developmental potential of goat embryos with a concomitant reduction in apoptosis rates.

Key words: *In vitro* maturation, goat, embryogenesis, programmed cell death, vitamin A

Caprine production is an economically relevant activity and for a few decades, techniques such as artificial insemination and embryo transfer have been extensively used. More recently, technologies based on *in vitro* production (IVP) of caprine embryos have received increasing attention to further improve genetic gain in breeding schemes, and to generate cloned and/or transgenic goats (Keefer et al., 2001; Baldassarre et al., 2004; Chiamenti et al., 2010, 2013). Despite these advances, caprine IVP-based technologies are hampered by low efficiency (Bal-

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dassarre and Karatzas, 2004), which is partly due to the limited understanding of species-specific requirements for *in vitro* oocyte maturation, fertilisation, and embryo culture.

A widely used approach to increase oocyte competence and embryonic development *in vitro* is to provide essential nutrients or survival factors. In goats, several reports have demonstrated that factors such α-lipoic acid, activin-A and retinoids enhance embryonic development when supplemented during oocyte maturation and/or embryo culture (Bormann et al., 2003; Chiamenti et al., 2010; Kwong et al., 2012; Zhang et al., 2013; Hammami et al., 2014). Vitamin A and its physiological metabolites, collectively known as retinoids, play important roles in embryonic morphogenesis and reproductive physiology as a mitogenic and differentiation stimulus (Hofmann and Eichele, 1994). However, the data regarding retinoids for caprine IVP are conflicting. Duque et al. (2002*b*) reported that addition of 9-cis-retinoic acid is beneficial to embryonic development in a quantitative and qualitative manner. Similar results were described by Lima et al. (2006) in bovine and Chiamenti et al. (2010) in caprine embryos, due to the fact that retinoids synchronise cellular events that trigger oocyte maturation, increase oocyte ability to be fertilised and potentiate further preimplantation embryonic development (Duque et al., 2002*a*; Lima et al., 2004; Lima et al., 2006; Chiamenti et al., 2010, 2013). However, Cavalcanti Neto (2004) did not observe any effect of retinyl acetate or 9-cis-retinoic acid on caprine oocyte nuclear and cytoplasmatic maturation. The understanding of the mechanism underlying retinoid treatment may guide future research in order to address this controversy.

The objective of the present study was to test if the addition of retinyl acetate (RAc) or 9-cis-retinoic acid (RA) during the *in vitro* maturation (IVM) of oocytes contributes to apoptosis inhibition in caprine oocytes and embryos.

Materials and methods

Oocyte retrieval and in vitro *maturation*

Caprine ovaries were obtained at local slaughterhouses (Pernambuco State, Brazil), and transported to the laboratory at 30 °C in 0.9% NaCl solution containing 30 μg mL^{-1} of gentamicin sulphate. Cumulus-oocyte complexes (COC) were recovered from 2- to 6-mm follicles with an 18 G needle in washing medium [8.0 mg of sodium bicarbonate, 45.0 mg of glucose, 5.6 mg of sodium pyruvate, 11.9 mg of HEPES, 2.5 mg of gentamicin sulphate and 20.0 mg of polyvinyl alcohol (PVA)] in 50 mL of TALP.

Oocytes were selected, washed and placed in maturation medium covered with sterile paraffin oil (Sigma-Aldrich) as previously described by Chiamenti et al. (2010). The maturation medium consisted of TCM-199 (Sigma-Aldrich) supplemented with 50 μg mL^{-1} of sodium pyruvate, 2.6 mg mL^{-1} sodium bicarbonate,

50 μg mL^{-1} of gentamicin sulphate, 5.0 μg mL^{-1} Pluset® and 1 mg mL^{-1} PVA. Oocytes were randomly distributed into the following groups: control (n = 1380), 0.3 μM retinyl acetate (RAc) (n = 1334), and 0.5 μM 9-cis-retinoic acid (RA) (n = 1358) in ten replicates. Furthermore, oocytes were incubated at 39 ºC with 5% CO_2 at saturated humidity for 24 h. After maturation and analysis of cumulus expansion, oocytes were randomly selected for caspase activity assay (n = 100), DNA fragmentation test (TUNEL assay, n = 100), and the remaining oocytes were destined for *in vitro* fertilisation (IVF).

In vitro *fertilisation*

Caprine IVF was performed as described earlier (Cavalcanti Neto, 2004; Chiamenti et al., 2010). Briefly, 0.1 mL of freshly collected semen was deposited in 1.5 mL of modified defined medium (mDM) (Keskintepe et al., 1998; Chiamenti et al., 2010). Viable sperm cells were selected by the swim-up method. After 45 min, 0.8 mL was aspirated from the upper layer and centrifuged at 350 *g* for 10 min. The pellet was supplemented with 200 μL of mDM containing 10 μg mL^{-1} of heparin.

Oocytes were assessed for cumulus expansion before IVF, and COC that did not expand were discarded. Pools of 25 oocytes were transferred to mDM containing a final sperm suspension of 2.0×10^6 spermatozoa mL^{-1} under sterile paraffin oil. Gametes were co-incubated for 18 h.

In vitro *embryo culture*

Presumptive zygotes were denuded and transferred to Potassium Simplex Optimized Medium (KSOM) and co-cultured on a monolayer of oviduct cells (Chiamenti et al., 2010). After 48 h of embryo culture, non-cleaved zygotes were removed and 30% of embryo culture medium was replaced with fresh KSOM. Embryonic development was recorded at day 3 (D3) and day 8 (D8). D8 blastocysts were analysed for caspase activity and DNA fragmentation (TUNEL assay).

Caspase activity

Caspase activity was determined as described earlier (Paula-Lopes and Hansen, 2002*a,b*; Roth and Hansen, 2004). Briefly, oocytes were denuded and washed in PBS supplemented with 1 mg mL^{-1} polyvinylpyrrolidone (PBS-PVP) and incubated in PBS-PVP containing 5 μM of PhiPhiLux-G1D2 for 40 min at 39 °C (Caspase-3 Intracellular Activity Assay Kit I, Calbiochem-Merck, Darmstadt, Germany). Moreover, oocytes were washed in PBS-PVP and caspase enzymatic activity was determined by fluorescence microscopy. Oocytes and embryos were scored as positive or negative for caspase activity. Ten replicates were performed.

TUNEL assay

The TUNEL assay was performed as previously described, with minor modifications (Paula-Lopes and Hansen, 2002*a,b*; Roth and Hansen, 2004). Oocytes were denuded and fixed in 4% paraformaldehyde for 1 h at room temperature. Oocytes were washed in PBS-PVP and incubated in permeabilisation buffer (0.5% Triton X-100 containing 0.1% sodium citrate) for 1 h and stored at 4 °C. Samples were further washed in PBS-PVP and incubated in terminal deoxynucleotidyl transferase enzyme solution (TUNEL) (*in situ* Cell Death Detection Kit, Fluorescein, Roche, Mannheim, Germany) for 1 h at 37 °C, were then washed in PBS-PVP and incubated with DNA-specific stain DAPI for 15 min. Oocytes were washed once more in PBS-PVP, transferred to slides with coverslips, and evaluated by fluorescence microscopy. Oocytes and embryos were scored as positive or negative for DNA fragmentation based on TUNEL. Ten replicates were performed.

Statistical analysis

Analysis of variance (ANOVA) by minimum squares method was used using PROC GLM (for fixed variables) and PROC MIXED (for fixed and random variables) from the SAS STAT package (SAS institute, Cary, NC). Before running the analysis, rough data were scored for ANOVA requirements (variables homogeneity and residues normality). Dependent and independent variables were established based on the experimental design. Statistical models considered all main effects. Differences with 5% probability were considered significant.

Results

In order to investigate the effect of retinoid addition on the incidence of apoptosis during caprine embryonic development, *in vitro* embryo production was performed by adding RAc or RA to oocyte IVM medium (Table 1). A defined IVM medium was used by replacing FBS with PVA, aiming to rule out any influence of serum, due to its complex and variable composition. Moreover, RAc and RA enhanced embryonic development at cleavage and blastocysts stages (Table 1). No difference was observed in embryo yields from the RAc and RA groups.

The TUNEL assay was used to detect DNA fragmentation in oocytes and embryos (Table 2), and the incidence of DNA fragmentation in oocytes was similar between retinoid-treated groups, but lower than in the control (Table 2). When blastocysts were analysed, the RAc and RA groups had less DNA fragmentation than the controls.

Table 1

In vitro embryo production using goat oocytes supplemented with retinyl acetate (RAc) or 0.5 μM 9-cis-retinoic acid (RA)

Group	Oocytes for maturation (n)	Oocytes for fertilisation (n)	Cleaved n (mean ± SD)	Blastocysts n (mean ± SD)
Control	698	412	107 (24.45 ± 2.32)[a]	10 (05.32 ± 0.81)[a]
RAc	816	532	170 (29.96 ± 1.62)[b]	18 (07.94 ± 0.93)[b]
RA	863	561	179 (30.12 ± 1.51)[b]	19 (07.36 ± 1.02)[b]

SD = standard deviation. The experiments were repeated ten times. [a,b]Values with different superscript letters are significantly different ($P < 0.05$)

Table 2

Effect of retinyl acetate (RAc) and 0.5 μM 9-cis-retinoic acid (RA) supplementation during oocyte maturation on DNA fragmentation in goat oocytes and blastocysts

Group	Oocytes (n)	TUNEL-positive oocytes n (mean ± SD)	Blastocysts (n)	TUNEL-positive blastocysts n (mean ± SD)
Control	100	09 (08.20 ± 0.78)	5	1 (08.70 ± 1.05)[a]
RAc	100	06 (05.60 ± 0.52)	9	1 (04.80 ± 0.51)[b]
RA	100	07 (06.40 ± 0.69)	10	1 (05.40 ± 0.69)[b]

SD = standard deviation; terminal deoxynucleotidyl transferase enzyme solution (TUNEL). The experiments were repeated ten times. [a,b]Values with different superscript letters are significantly different ($P < 0.05$)

Caspase activity was initially investigated in oocytes (Table 3). No difference was observed in the incidence of caspase activity in oocytes within groups. Moreover, caspase activity was more frequent in control than in RAc and RA blastocysts (Table 3).

Table 3

Caspase activity in goat oocytes and blastocysts after supplementation with retinyl acetate (RAc) or 0.5 μM 9-cis-retinoic acid (RA) during oocyte maturation

Group	Oocytes (n)	Caspase-positive oocytes n (mean ± SD)	Blastocysts (n)	Caspase-positive blastocysts n (mean ± SD)
Control	100	08 (07.20 ± 0.91)	5	1 (08.35 ± 0.71)[a]
RAc	100	07 (06.60 ± 0.68)	9	1 (05.05 ± 0.62)[b]
RA	100	08 (07.30 ± 0.67)	9	1 (05.75 ± 0.22)[b]

SD = standard deviation. The experiments were repeated ten times. [a,b]Values with different superscript letters are significantly different ($P < 0.05$)

Discussion

The effect of retinoids *in vivo* has been extensively described (Shaw et al., 1995; Hidalgo et al., 2002), but their benefit to caprine embryonic development *in vitro* remains controversial. Despite this, the data described here show that retinoids increase oocyte competence *in vitro,* in accordance with reports in several species such as the porcine, bovine and canine species (Almiñana et al., 2008; Deb et al., 2011, 2012; Liang et al., 2012).

Furthermore, it was sought to investigate if caprine oocyte and embryo quality is also improved by retinoids, measured by the incidence of apoptosis. Several efforts have been made to find connections between the characteristics of caprine oocytes and their apoptosis incidence as well as the influence of the latter on embryonic development. It has been shown that the diameter of healthy oocytes correlates with the incidence of apoptosis (Anguita et al., 2009), follicle size correlates with DNA fragmentation in oocytes (Romaguera et al., 2010), and glucose-6-phosphate dehydrogenase (G6PDH) activity of goat oocytes (determined by brilliant cresyl blue staining) enhances embryonic development and increases Bcl-2 expression in blastocysts (Abazari-Kia et al., 2014).

In this study, two markers of apoptosis were chosen to characterise apoptosis, namely DNA fragmentation and caspase activation, in order to avoid misleading interpretations due to other types of cell death during preimplantation development (Gjørret et al., 2003).

Apoptosis was characterised in bovine oocytes by cytoplasmic condensation and fragmentation followed by apoptotic body formation with or without DNA fragmentation (Perez et al., 1999; Mem et al., 2003). This DNA damage detected by the TUNEL assay is not always caused by the activity of effector caspases (Van Blerkom and Davis, 1998; Xu et al., 2001). Activation of apoptosis mechanisms upstream of caspase activation leads to oocyte apoptosis (Roth and Hansen, 2004). Collectively, the association of DNA damage and caspase 3 detection can successfully be used to describe apoptosis in oocytes and embryos.

The validity of the association between these two apoptosis markers is in accordance with findings described in the present report, where DNA fragmentation in caprine oocytes did overlap with caspase activity in all experimental groups. Caspase-positive oocytes displayed a broad cytoplasm without condensation, a fact that is considered normal, and were observed mainly in oocytes treated with retinoids. To the best of our knowledge, this is the first report describing changes in IVP conditions that increase embryo quantity and improve embryo quality by reducing apoptosis.

Despite the pleiotropic nature of the role of retinoids during development, it is becoming clear that cumulus cells play a major role in mediating the effects of retinoids during *in vitro* oocyte maturation. Gene expression analysis has revealed transcriptional changes in cumulus cells in response to retinoids that are

related to the prevention of apoptosis: up-regulation of midkine (Gómez et al., 2003), down-regulation of aldo-keto reductase family 1 member B1 (AKR1B1), a gene potentially associated with pregnancy failure (Deb et al., 2012), and down-regulation of proto-oncogenes and extracellular signal-regulated kinases 1/2 (ERK1/2) and Janus kinase (JNK) signalling pathways (Deb et al., 2012). Retinoid-treated cumulus cells also express lower levels of initiator and effector caspases, TNF-α, TNFR1, BAX (Deb et al., 2012; Liang et al., 2012) and higher levels of BCL-2 (Deb et al., 2012). Despite these benefits from cumulus cells, retinoid treatment did not reduce the incidence of oocyte apoptosis, due to the short interval between treatment and assessment, or because of an unknown factor. Retinoid treatment before maturation could be more effective against oocyte apoptosis (Duque et al., 2002*a*). The increase in blastocyst quantity and quality after retinoid treatment during oocyte maturation is in accordance with a long-term effect model.

Retinoids, when supplemented during embryo culture, require co-culture systems, at least under some experimental conditions, in order to enhance embryonic development (Lima et al., 2004; Chiamenti et al., 2010, 2013). This fact suggests that this paracrine signalling could be potentially useful for increasing embryonic development *in vitro* in other species and should be the focus of future research aimed at describing the molecular basis of retinoid embryotrophic properties *in vitro*.

Although more research is needed to mechanistically elucidate all the roles played by retinoids in enhancing caprine embryonic development *in vitro*, we conclude that at least part of this benefit comes from the inhibition of programmed cell death as evidenced by the reduction in the incidence of apoptosis markers.

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