Effect of urokinase type plasminogen activator on in vitro bovine oocyte maturation

Roldán-Olarte Mariela 1,2, Maillo Verónica 1, Sánchez-Calabuig María Jesús 1, Beltrán-Breña Paula 1, Rizos Dimitrios 1 and Gutiérrez-Adán Álfonso 1

1 Department Reproducción Animal, INIA, Madrid, Spain and 2 Instituto Superior de Investigaciones Biológicas (INSIBIO), CONICET-Universidad Nacional de Tucumán (UNT), San Miguel de Tucumán, Argentina

Correspondence should be addressed to M Roldán-Olarte; Email: emroldanolarte@fbqf.unt.edu.ar

Abstract

This study examines the impacts of the urokinase-type plasminogen activator (uPA) on the in vitro maturation (IVM) of bovine oocytes. Cumulus-oocyte complexes in IVM medium were treated with uPA, amiloride (an uPA inhibitor), dimethyl sulfoxide (DMSO) or left untreated (control group). After 24 h of IVM, oocytes were recovered for testing or were in vitro fertilized and cultured to the blastocyst stage. The factors examined in all groups were: (i) oocyte nuclear maturation (Hoechst staining); (ii) oocyte cytoplasmic maturation (cortical granules, CGs, distribution assessed by LCA-FITC); (iii) oocyte and cumulus cell (CC) gene expression (RT-qPCR); and (iv) embryo development (cleavage rate and blastocyst yield). Oocytes subjected to uPA treatment showed rates of nuclear maturation and CG distribution patterns similar to controls (P > 0.05), whereas lower rates of oocyte maturation were recorded in the amiloride group (P < 0.05). Both in oocytes and CC, treatment with uPA did not affect the transcription of genes related to apoptosis, cell junctions, cell cycle or serpin protease inhibitors. In contrast, amiloride altered the expression of genes associated with cell junctions, cell cycle, oxidative stress and CC serpins. No differences were observed between the control and uPA group in cleavage rate or in blastocyst yield recorded on Days 7, 8 or 9 post-insemination. However, amiloride led to drastically reduced cleavage rate (28.5% vs 83.2%) and Day 9 embryo production (6.0% vs 21.0%) over the rates recorded for DMSO. These results indicate that the proteolytic activity of uPA is needed for successful oocyte maturation in bovine.

Introduction

The structural integrity of the cumulus cell (CC) extracellular matrix (ECM) is essential for oocyte maturation (Zhuo & Kimata 2001, Salustri et al. 2004). Several cumulus proteins linked to hyaluronan are present around CCs and have been attributed a role in oocyte expansion and oocyte maturation (Lu et al. 2013). The task of ECM remodeling is the responsibility of proteolytic enzymes. As a proteolytic system, the plasminogen activation system has the capacity to act on a wide broad spectrum of substrates (Collen 1980, Saksel 1985, Liu et al. 2004, Deryugina & Quigley 2012). Plasminogen is an extracellular proenzyme that is abundant in blood plasma and most extracellular fluids (Plow et al. 1995), especially follicular and oviductal fluids (Beers 1975, Mondéjar et al. 2012). Plasminogen is activated to plasmin by two types of plasminogen activators: urokinase-type (uPA) and tissue-type (tPA). Both activators are produced by bovine cumulus-oocyte complexes during their in vitro maturation (Park et al. 1999). A role for uPA has been established in the pericellular proteolysis and is required for cell migration and tissue remodeling (Andreasen et al. 2000, Deryugina & Quigley 2012). Binding to specific receptors (uPAR) localizes uPA activity at the cell surface (Blasi & Sidenius 2010, Smith & Marshall 2010). The activity of uPA is controlled by the specific inhibitor type 1 (PAI-1), a member of the serpin proteinase inhibitor superfamily (Potempa et al. 1994). The uPA substrate, plasminogen, has been detected in the plasma membrane and zona pellucida of hamster oocytes (Jiménez-Díaz et al. 2002), immature pig oocytes (Roldán-Olarte et al. 2005) and in in vitro matured porcine and bovine oocytes (Mondéjar et al. 2012). Recently, it was observed that the gene that codifies uPA (PLAU) is only expressed in the CCs of immature or in vitro matured COCs, while PLAUR and PAI-1 are expressed in both CCs and in immature and in vitro matured oocytes (García et al. 2016). These authors propose that the plasminogen activation system could play a critical role in the oocyte maturation process. When tPA activity was determined in cortical
granule extracts (Rekkas et al. 2002), it was related to post-fertilization events such as the cortical reaction and the block of polyspermy at the zona pellucida (Mondèjar et al. 2012). Plasmin, the central protease of this system, is required for physiological processes such as ovulation (Liu et al. 2004), CC expansion (Liu et al. 2004), oocyte maturation (Dow et al. 2002), fertilization (Smokovitis et al. 1992, Huarte et al. 1993), zona reaction (Zhang et al. 1992, Cannon & Menino 1998, Rekkas et al. 2002) and embryo hatching (Menino & Williams 1987, Kaeukuahiwi & Menino 1990).

Despite numerous works addressing the role of the plasminogen activation system during the initial stages of reproduction (Papanikolaou et al. 2008, Coy et al. 2012, Grullon et al. 2013, Krania et al. 2015a,b), the contributions of each of its components to each stage of in vitro embryo production have not been well established. What has been established is that the inhibition of endogenous uPA compromises cumulus expansion during the in vitro maturation of cumulus–oocyte complexes (COCs) in mice and humans (Lu et al. 2013). The latter authors reported that the over-expression of SERPINE2, or exogenous supplementation with high levels of SERPINE2 impaired cumulus expansion and oocyte maturation. This protein is a member of the serpin family, a group of proteins that inhibit serine proteases such as thrombin, uPA, plasmin and trypsin. Amiloride, a specific inhibitor of uPA, produced a similar effect on cumulus expansion. Amiloride competitively inhibits the catalytic activity of uPA while it has no effects on tPA (Vassalli & Belin 1987). These different effects can be attributed to structural differences between the two activator types (Jankun & Skrzypczak-Jankun 1999, Zhu et al. 2007).

The present study was designed to address the effects of uPA on in vitro bovine oocyte maturation by separately determining the impacts of adding uPA or a specific inhibitor of uPA (amiloride) to the in vitro maturation (IVM) medium.

Materials and methods

Unless stated otherwise, all chemicals were purchased from Sigma-Aldrich Química.

Oocyte collection and IVM

Immature COCs were obtained by aspirating follicles (2–8 mm diameter) from the ovaries of mature heifers (i.e. at least one corpus luteum or remained scars from previous ovulations in one or both ovaries) collected at slaughter from a local abattoir (Transformación Ganadera de Leganés S.A., Madrid, Spain). Class 1 and class 2 COCs (homogeneous cytoplasm and intact CCs) were matured for 24 h in 500 µL of maturation medium, TCM 199 supplemented with 10% v/v fetal calf serum (FCS) and 10 ng/mL epidermal growth factor in four-well dishes, in groups of 50 COCs per well at 38.5°C under an atmosphere of 5% CO2 in air, with maximum humidity (Rizos et al. 2002). Each experiment consisted of four groups of 50 COCs, in accordance to each treatment given at the start of IVM: (I) no treatment (control); (II) uPA (10 nM, Sigma, U0633-25UG; solubilized in sterile-filtered water, to obtain a stock solution of 1 µM); (III) dimethyl sulfoxide (DMSO, 0.02% as the amiloride vehicle); and (IV) amiloride (100 µg/mL, Sigma, A0370000; solubilized in DMSO to obtain a stock solution of 50 mg/mL).

The concentration of uPA was based on the findings of other studies in which the effect of uPA on expression levels of C-FOS in oviductal epithelial cells (García et al. 2014) and ovarian cancer cells (Dumler et al. 1994) was analyzed. The amiloride concentration used was selected according to the findings of Lu et al. (2013) and Ding et al. (2012). The first study evaluated the effect of 0.3 mM amiloride on the expansion of murine and human COCs and the latter study reported that 0.01, 1 and 1 mM amiloride decreases PLAU expression levels in human gastric cancer cell lines after 24 h of incubation (Ding et al. 2012).

COCs matured under different conditions were employed to evaluate: developmental competence after in vitro fertilization, nuclear maturation, cortical granules (CGs) distribution and gene expression in oocytes and CCs. From each group, 10 oocytes were employed to the evaluation of nuclear maturation and CG distribution, 10 COCs to the gene expression analysis and the remaining were destined to in vitro fertilization and posterior embryo development. Four replicates of each experiment were set up.

Sperm preparation and in vitro fertilization (IVF)

Frozen semen straws (0.25 mL) from an Asturian Valley bull previously tested for IVF (ASEAVA, Asturias, Spain) was thawed at 37°C in a water bath for 1 min and centrifuged for 10 min at 280 g through a gradient of 1 mL of 40% and 1 mL of 80% Bovipure (Nidacon Laboratories AB, Göthenborg, Sweden Bovipure) according to the manufacturer's instructions. The sperm pellet was isolated and washed in 3 mL of Boviwash (Nidacon Laboratories AB) by centrifugation at 280 g for 5 min. The pellet was re-suspended in the remaining 300 µL of Boviwash. Sperm concentration was determined and adjusted to a final concentration of 1 x 10^6 sperm/mL for IVF. Gametes were coincubated for 18–22 h at 38.5°C in four-well plates in groups of 50 COCs per well under an atmosphere of 5% CO2 in air and maximum humidity. Each well contained 500 µL of Tyrode's fertilization medium containing 25 mM bicarbonate, 22 mM Na-lactate, 1 mM Na-pyruvate and 6 mg/mL fatty acid-free bovine serum albumin (BSA) supplemented with 10 mg/mL heparin sodium salt (Calbiochem) (Lopera-Vásquez et al. 2016).

In vitro culture of presumptive zygotes

At approximately 20 h post-insemination (hpi), presumptive zygotes were denuded of CCs by vortexing for 3 min and then cultured in groups of 25 in 25 µL droplets (control: n = 129; uPA: n = 205; DMSO: n = 137; amiloride: n = 209) of synthetic oviductal fluid (SOF) containing 4.2 mM sodium lactate (L4263), 0.73 mM sodium pyruvate (P4562),
30 µL/mL BME amino acids (B6766), 10 µL/mL minimum essential medium (MEM) amino acids (M7145) and 1 µg/mL phenol red (P0290) under mineral oil at 38.5°C under an atmosphere of 5% CO₂, 5% O₂ and 90% N₂, as the embryo culture is routinely performed (Lopera-Vásquez et al. 2016). SOF was supplemented with 5% FCS (Gutiérrez-Adán et al. 2001, Rizos et al. 2008).

Embryo development and quality

Cleavage rates were recorded on Day 2 (48 hpi) and cumulative blastocyst yields on Days 7, 8, and 9 post-insemination under a stereomicroscope. Four different experiments, each of them with 4 experimental conditions were performed.

Nuclear maturation and cortical granules distribution patterns

Nuclear maturation and CG distribution, as one parameter of cytoplasmic oocyte maturation, were assessed by confocal microscopy following a method described previously (Coy et al. 2005). Briefly, first 10 in vitro matured COCs from each treatment were suspended in 100 µL of phosphate-buffered saline (PBS) without calcium or magnesium supplemented with 1% BSA and their CCs were removed by gently pipetting. Next, oocytes were treated with 0.5% w/v pronase in PBS to digest the zona pellucida. Zona-free oocytes were washed in PBS three times and fixed in 3.7% w/v buffered neutral parafomaldehyde solution (pH 7.2–7.4) for 30 min at room temperature and treated with permeabilization solution (0.01% v/v Triton X-100 in PBS) for 10 min. The oocytes were then treated for 30 min with blocking solution (7.5% w/v BSA in PBS) and incubated in 100 µg/mL FITC-labeled Lens culinaris agglutinin (LCA-FITC Sigma, L9262) for 40 min in a dark chamber. Chromatin was stained with 1 µg/mL Hoechst 33342 for 5 min. After staining, oocytes were washed, mounted in 2 µL of mounting medium (50% w/v PBS, 50% v/v glycerol (Sigma G-S150), 0.0025 µg/mL Hoechst) between a coverslip and a glass slide and sealed with nail polish. Slides were examined using a laser-scanning confocal microscope (MRC 175 1024, Bio-Rad) equipped with an argon laser excited at 488 nm and whose detection spectrum is 515–530 nm. Nuclear maturation was observed in an epifluorescence microscope (Nikon 141731) equipped with a fluorescent lamp (Nikon HB-10104AF) and UV-1 filter and oocytes were classified as follows: germinal vesicle stage (GV). For the nuclear maturation, all the nucleus and polar bodies were evaluated, oocytes (control: n=55; uPA: n=41; DMSO: n=59; amiloride: n=33) were classified as GV; germinal vesicle stage (nucleus well-defined) MI: metaphase I (first metaphasic plate visible); or MI: metaphase II (nucleus mature, represented by the presence of first polar body, observed before ZP dissolution, or second metaphasic plate). As a parameter of cytoplasmatic maturation, CGs were analyzed (control: n=39; uPA: n=28; DMSO: n=39; amiloride: n=35 oocytes) and the distribution of cortical granule was classified as three types (type I, distributed in clusters; type II, dispersed and partly clustered; and type III, small CG arranged at the periphery) (Hosoe & Shioya 1997). Four replicates were carried out.

Oocytes and cumulus cells for gene expression analysis

After 24h of IVM, pools of 10 COCs were collected from each treatment group (four replicates) and CCs physically separated from oocytes by gentle pipetting. Oocytes, in pools of 10 per treatment group, were washed in PBS, snap frozen in liquid N₂ and stored at −80°C until mRNA extraction. Their corresponding CCs were also washed in PBS, centrifuged at 10,000g and then snap frozen in liquid N₂ and stored at −80°C until mRNA extraction.

Gene expression

For gene expression studies, pools of 10 oocytes and their corresponding CCs from each experimental group were analyzed separately in 4 replicates.

Poly(A) RNA was extracted using the Dynabeads mRNA DIRECT Micro Kit (Ambion, Thermo Fisher Scientific) according to instructions with minor modifications (Bermejo-Alvarez et al. 2008). Immediately after extraction, the reverse transcription (RT) reaction was run according to the manufacturer’s instructions (Epicentre Technologies Corp., Madison, USA) using poly(T) primer, random primers and Moloney murine leukemia virus (MMLV) reverse transcriptase.

The tubes were heated to 70°C for 5 min to denature the secondary RNA structure and then the RT mix was completed with the addition of 50 units of reverse trancriptase. The tubes were incubated at 25°C for 10 min to induce the annealing of random primers, followed by 37°C for 60 min to allow the RT of RNA, and finally 85°C for 5 min to denature the enzyme. All qPCR reactions were run in duplicate in the Rotorgene 6000 Real Time Cycler TM (Corbett Research, Sydney, Australia). In each run, 2 µL aliquots of each sample were added to the PCR mix (GoTaq qPCR Master Mix, Promega) containing specific primers to amplify the genes contained in Table 1. The selection of genes to be evaluated in oocytes and CC was carried out considering that they are representatives of key processes, i.e. apoptosis (BAX, BCL2, TP53, SHC1), cell junctions (GJA1, TJP1), oxidative stress (SOD2, GPX1), cell cycle (CCNB1), oocyte quality (GDF9, BMP15) and serpin protease inhibitors (SERPIN1, SERPINA5) as previously described by several authors (Feuerstein et al. 2007, Assidi et al. 2008, Assou et al. 2010, Bermejo-Alvarez et al. 2010, Dovolou et al. 2014, Blaha et al. 2015). Primer sequences and sizes of the amplified fragments of all transcripts are provided in Table 1. Cycling conditions were 94°C for 3 min followed by 35 cycles of 94°C for 15s, 56°C for 30s, 72°C for 10s and 10s of fluorescence acquisition. Each pair of primers was tested to obtain efficiencies close to 1. The comparative cycle threshold (Ct) method was used to quantify expression levels (Schmittgen & Livak 2008). In each cycle, fluorescence was acquired at a temperature higher than the melting temperature of primer dimers to avoid primer–dimer artifacts (specific for each product, 76–86°C). The threshold cycle or the cycle during the log-linear phase of the reaction at which fluorescence increased above background was determined for each sample. Within this region of the amplification curve, a difference of one cycle is equivalent to a doubling of the amplified PCR product. According to the comparative Ct method, the ACt value was determined by subtracting the endogenous control

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Ct value (mean for H2AFZ and ACTB) for each sample from each gene Ct value of the sample. To calculate ΔΔCt, the highest treatment ΔCt value (i.e. the treatment with the lowest target expression) was used as a constant to subtract from all other ΔCt sample values. Fold changes in the relative gene expression of the target were calculated using the formula $2^{-\Delta\Delta C_{t}}$ (Livak & Schmittgen 2001).

**Results**

**Developmental competence of bovine oocytes in vitro matured in the presence of uPA or amiloride**

The results on cleavage and embryo development are shown in Fig. 1. Supplementation of uPA to IVM medium did not affect cleavage rate compared to control and DMSO groups (85.7 ± 4.1% vs 80.9 ± 1.4% and 83.2 ± 2.1% respectively), while supplementation of amiloride decreased significantly (28.5 ± 5.2%).

Blastosyst yield on Day 7 was similar for uPA and control groups (18.6 ± 1.5% and 17.4 ± 4.2%) while significantly lower to DMSO and amiloride (8.0 ± 2.6% vs 1.3 ± 1.0%). Similarly, for Days 8 and 9 blastocyst yield was no different for uPA, control and DMSO groups (Day 8: 26.5 ± 3.4%; 25.2 ± 2.9%; 21.0 ± 3.8% respectively) while it was significantly decreased for amiloride group (Day 8: 5.3 ± 2.2% and Day 9: 6.0 ± 1.9%).

**Statistical analysis**

All statistical tests were performed using SigmaStat (Jandel Scientific, San Rafael, CA, USA) and InfoStat (InfoStat 2015, http://www.infostat.com.ar) statistical softwares. Data for cleavage rates, blastocyst yields, nuclear maturation, CG distribution patterns and relative mRNA abundance were compared by one-way analysis of variance (ANOVA). When they showed normality, significant differences between the mean values were determined (LSD Fisher’s test, $P<0.05$). In addition, t-test was also applied to pairwise comparisons.

### Table 1  Primers used for RT-qPCR.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Primer sequence (5′→3′)</th>
<th>Fragment size (bp)</th>
<th>GenBank access no.</th>
</tr>
</thead>
</table>
| ACTB        | Actin, beta                       | Forward: GAGAAGCTCTGCTACGTGG  
Reverse: CCAGGACCCCGGTGTTGG  | 264 | AF191490.1 |
| BAX         | BCL2-Associated X Protein         | Forward: CTGGAGCAGTGGCCCTGAGA  
Reverse: ATTCGAGAAAGGAGGGTCGTC  | 300 | NM_001166486.1 |
| BCL2        | B-Cell CLL/Lymphoma 2             | Forward: GAGCCTGTTGGGCTACGCTTC  
Reverse: TAAGGGCACAGGAGGGTCGTC  | 517 | BC147863.1 |
| BMP15       | Bone Morphogenetic Protein 15     | Forward: ATATGCTCATCACCAGAGACC  
Reverse: TAAAGCATGAGCCAGGTACA  | 72  | NM_001031752.1 |
| CCNB1       | Cyclin B1                        | Forward: TCGTGTCGCTTTCTAATGCTCC  
Reverse: CGAGTGTGCACTATGGTTCTATTAGTCA  | 332 | NM_001045872.1 |
| GDF9        | Growth differentiation factor 9    | Forward: AGGCCGCTACTGCTTCTATAT  
Reverse: TTCCCTTTCAGGCTGAGGGCA  | 80  | NM_174681.2 |
| GJA1        | Gap junction protein, alpha 1     | Forward: GGCTCATCTGCTTCTATAT  
Reverse: AGAACACATGAGCCAGGTACA  | 142 | NM_174068.2 |
| GPX1        | Glutathione Peroxidase 1          | Forward: GCAACAGTGGCCGATCA  
Reverse: CTGGACCTTTTCGAAGAGCA  | 116 | NM_174076.3 |
| H2AFZ       | H2A histone family, member Z      | Forward: AGGCCGACTCAGCTGCTTGTG  
Reverse: CCAACCGAGCAATGGTGAGCTTG  | 209 | NM_174809 |
| SCH1        | SHC (Src Homology 2 Domain Containing) Transforming Protein 1 | Forward: GTGACGTTGGGAGAGAAC  
Reverse: GGTCCGCAAAAGGATCAC  | 334 | NM_001075305 |
| SERPINA5    | Serpin Peptidase Inhibitor, Clade A (Alpha-1 Antitrypsin)  
Member 5 | Forward: TGGAATGCGCTTGAAGAAA  
Reverse: ATAAAGCTGAACCCGCCCTTT  | 74  | NM_176466 |
| SERPINE1    | Serpin Peptidase Inhibitor, Clade E (Nexin, Plasminogen Activator Inhibitor Type 1, Member 1) | Forward: CAGGCAGGTCTTCCAGGTTC  
Reverse: ACCTCAACTTCCACCTTCTGCAG  | 77  | NM_174137 |
| SOD2        | Superoxide Dismutase 2, Mitochondrial (former MnSOD) | Forward: GCTTATCGATGGCTTGG  
Reverse: AAAGTAAAGCCAGTTCCTCC  | 101 | S67818.1 |
| TJP1        | Tight Junction Protein 1          | Forward: AACATCCGACTCTGCTGCA  
Reverse: CCAACAGCGGCAGTAAA  | 255 | XM_010817146.1 |
| TP53        | Tumor Protein P53                  | Forward: CTGTCCTGCTGCTA  
Reverse: GGATCCAGGATAGTGGAGC  | 364 | NM_174201.2 |

*Ct value (mean for H2AFZ and ACTB) for each sample from each gene Ct value of the sample. To calculate ΔΔCt, the highest treatment ΔCt value (i.e. the treatment with the lowest target expression) was used as a constant to subtract from all other ΔCt sample values. Fold changes in the relative gene expression of the target were calculated using the formula $2^{-\Delta\Delta C_{t}}$ (Livak & Schmittgen 2001).*
Nuclear maturation

Nuclear maturation was unaffected by the presence of 10 nM uPA. Similar MII rates were recorded for the uPA and control groups (62.7 ± 10.7% vs 62.7 ± 8.2% respectively). However, the addition of amiloride to the IVM medium led to a drastic increase in number of oocytes with intact GV (Fig. 2) indicating that a higher percentage of oocytes remained arrested or had not initiated meiosis (83.3 ± 6.6% vs 14.9 ± 8.0% in DMSO group, \( P < 0.05 \)).

Cortical granules distribution patterns

No significant differences in CG distributions were observed between the uPA and control groups (type III CG distribution observed in 32.9 ± 7.0% vs 51.9 ± 16.5% respectively; Fig. 3). Interestingly, significant differences were detected between amiloride and control groups. As shown in Fig. 3, most oocytes matured in the presence of amiloride showed a type I CG distribution pattern compared to oocytes in the control DMSO group (75.0 ± 6.0% vs 14.4 ± 10.9% respectively), while no oocytes displayed a type III CG distribution (0.0% vs 25.4 ± 5.8% respectively; \( P < 0.05 \)). The presence of oocytes with type II CG distribution pattern was also lower under amiloride treatment (25.0 ± 1.6) than with uPA supplementation (31.0 ± 11.3), DMSO group (60.2 ± 10.4) and control (29.5 ± 10.4).

Effect of uPA and amiloride on gene expression levels in oocytes and cumulus cells

The presence of uPA in the IVM medium only affected the expression of \( SOD2 \) in oocytes while no differences were observed in CC. The relative abundance of \( SOD2 \) in oocytes was higher in the uPA group than control group (\( P < 0.05 \)) (Fig. 4A).

In contrast, the addition of amiloride during in vitro maturation affected the expression levels of several genes. Compared with controls, \( GPX1 \) and \( GJA1 \) were upregulated while \( CCNB1 \) and \( BMP15 \) were downregulated in oocytes (Fig. 5A), and \( BAX, BCL2, TP53, SHC1, TJ1, GJA1 \) and \( CCNB1 \) were upregulated, while \( SOD2, SERPINE1 \) and \( SERPINA5 \) were downregulated in CC (Fig. 5B).
Discussion

The role played by the plasminogen activation system in the initial steps of mammalian reproduction has been addressed by many researchers who have tried to elucidate its implication in the processes of gametogenesis (Liu et al. 1986, 2013), fertilization (Huarte et al. 1993, Mondejar et al. 2012), early embryonic development (Aflalo et al. 2005) and implantation (Whiteside et al. 2001). In the present study, we sought to determine whether uPA activity could affect the quality of oocytes used for in vitro embryo production, their developmental competence and the expression of candidate genes in oocytes and CC after IVM.

According to our findings, excess uPA during IVM did not affect oocyte maturation nor embryo development. In a preliminary experiment two different concentrations of uPA were evaluated, 10 and 50 nM. No significant differences were detected in the percentages of cleavage (78.0 ± 5.5 in control; 86.2 ± 3.8 with uPA 10 nM and 77.2 ± 1.1 with uPA 50 nM) nor blastocyst rate (23.9 ± 2.6 in control; 28.7 ± 3.1 with uPA 10 nM and 20.8 ± 0.8 with uPA 50 nM). Then, we decided to use 10 nM of uPA as previously reported (Dumler et al. 1994, García et al. 2014). Given that inhibitors of the plasminogen activation system exist in bovine COCs (Bieser et al. 1998, García et al. 2016), these could both in vivo and in vitro control the proteolytic activity of the system, diminishing the activity of exogenous uPA. Therefore, an excess of uPA activity could be controlled by inhibitors that maintain the balance of proteolytic activity initiated by uPA. This is in agreement with what has been described by other authors (Krania et al. 2015a), who analyzed the effect of the inclusion of exogenous uPA in the in vitro bovine embryo culture medium. They found that it does not have any effect on embryo yield and/or quality. These authors highlight the importance to the balance of activity between activators and inhibitors in a highly regulated manner, to ensure the correct embryo development. In the same extend during in vitro oocyte maturation, a well-balanced proteolytic activity must be of the importance to assure the oocyte developmental competence. To examine whether endogenous uPA is important for in vitro maturation, we blocked the catalytic activity of uPA present in the female gamete using amiloride. Our results indicate that amiloride inhibits the maturation of bovine oocytes at
the nuclear level as well as cytoplasmic level, taking into account the CG distribution. We thus inferred that the proteolytic activity of uPA is required for the processes of bovine oocyte maturation and CC expansion, in agreement with observations in mouse COCs (Lu et al. 2013). The main function of uPA is to generate plasmin through activation of plasminogen near the plasma membrane of cells that express uPAR (Blasi & Sidienius 2010). The presence of uPAR and plasminogen has been established in the female bovine gamete (Mondèjar et al. 2012, García et al. 2016), indicating that plasmin generation in bovine COCs could involve uPA bound to its receptor at the plasma membrane of the oocyte and CCs. By blocking uPA activity using amiloride, we observed that plasmin generation mediated by uPA is needed to ensure COC maturation. It has been reported that plasmin increases maturation rates when added 18h after in vitro maturation without affecting embryonic developmental rates (Papanikolaou et al. 2008). However, plasmin’s mechanism of action during oocyte maturation remains to be established.

To gain insight into the molecular mechanisms affected by the presence of uPA or amiloride during IVM, we examined, in both oocytes and CCs, the expression of candidate genes related to apoptosis (BAX, BCL2, TP53, SHC1), cell junctions (GJA1, TJP1), cell cycle (CCNB1), oxidative stress (SOD2, GPX1), oocyte quality (BMP15, GDF9) and serpin protease inhibitors (SERPINE1, SERPINA5). Although a limited number of genes were analyzed in this study, several changes were registered under different treatments carried out during COCs in vitro maturation, suggesting that certain cellular processes are affected, especially in the presence of amiloride. We observed that uPA does not provoked changes in the expression levels of these genes in CCs, while in oocytes, only SOD2 was upregulated, suggesting that uPA could protect against oxidative stress (Bermejo-Alvarez et al. 2010, Dovolou et al. 2014). In contrast, the presence of amiloride modified the expression of several of the genes examined in both oocytes and CCs.

In oocytes matured in the presence of amiloride, GJA1, related to cell junctions was upregulated respect to oocytes corresponding to the DMSO control group, indicating increased gap junctions between oocyte and CCs. On the contrary, CCNB1, involved in the regulation of cell cycle and BMP15 as a mechanism promoting the developmental competence of the oocytes was downregulated. The reduced expression of this gene has been reported to impair GV breakdown (Sánchez & Smitz 2012). A higher CCNB1 mRNA level has been linked to the greater activity of mitosis-promoting factor (Bermejo-Alvarez et al. 2010). CCNB1 translation regulates oocyte meiosis resumption (Levesque & Sirard 1996), and an abundance of its mRNA has been correlated with developmental competence in goats (Anguita et al. 2008). In our study, the downregulation of CCNB1 could indicate the reduced capacity of the oocyte to resume meiosis when matured in the presence of amiloride.

In CCs, the apoptosis-related genes (BAX, BCL-2, TP53 and SHC1) were upregulated when amiloride was added to the IVM medium. However, the BAX/BCL2 ratio was not significantly modified. Besides, it is known that BMP15 serves to maintain a low level of CC apoptosis (Hussein et al. 2005). Interestingly, amiloride downregulated oocyte BMP15 possibly reducing its antiapoptotic effect. These results suggest a tendency of CCs to initiate apoptosis. Nevertheless, in these cells CCNB1 was upregulated. The molecular mechanism whereby amiloride affects apoptosis and/or proliferation of CCs requires clarification. The expression of TJP1 and GJA1 involved in gap junction connections was markedly upregulated in CCs. Studies have shown that GJA1-mediated gap junction communication regulates oocyte meiosis resumption, and that lower levels of GJA1 in CCs are beneficial for oocyte maturation (Fair 2003, Edry et al. 2006). Indeed, this gene has been proposed as a potential marker of oocyte maturation (Li et al. 2015). Accordingly, the upregulation of TJP1 and GJA1 by amiloride noted here could be related to the observed inhibition of oocyte maturation.

We also detected the downregulation of SOD2 in CCs exposed to amiloride. This could reflect a certain vulnerability of COCs to oxidative stress, thereby influencing the subsequent developmental competence of the oocyte (Combelles et al. 2010). SERPINE1 and SERPINA5 were downregulated. Both genes code for serine protease inhibitors. SERPINA5 has been described as one of the most over-expressed genes in CCs after in vivo and IVM (Salhab et al. 2013, Blaha et al. 2015). This protein plays a role in the regulation of ECM degradation, coagulation, fibrinolysis, wound healing and fertility (Suzuki 2008, Meijers & Herwald 2011). In the present study, the downregulation of SERPINE1 and SERPINA5 observed in CCs treated with amiloride suggests the altered balance of ECM remodeling affecting CC expansion.

Although the mechanism of amiloride action in bovine COCs has not been evaluated, its action as inhibitor of the uPA proteolytic activity would be responsible of this effect, as suggested by Lu et al. (2013). Even though the principal action of uPA is to proteolytically activate plasminogen to plasmin, several molecules could be substrate of plasmin or uPA itself, such as certain members of metalloproteinases (Zhao et al. 2008). Recent studies demonstrated that uPA is able to activate the epithelial sodium channel (ENaC), by the proteolysis of γ ENaC (Ji et al. 2015), a known target of amiloride (Kashlan et al. 2005). This is in agreement with the dual effects of amiloride, since it can produce directly the ENaC inhibition and indirectly through its novel effects on uPA activity with the consequent attenuation of posttranslational ENaC activation, proposed by other authors (Svenningsen et al. 2015, Warnock 2015, Blaha et al. 2015).
Taking into account all these evidences, it is probable that a regulated proteolytic activity of uPA is necessary to ensure the quality of oocyte maturation process and that amiloride disrupt this equilibrium.

In conclusion, although uPA supplementation during in vitro maturation did not affect oocyte maturation or early embryo production, blockage of endogenous uPA activity by amiloride unveiled an important role for uPA in bovine oocyte maturation. Further work is needed to clarify the biological and molecular mechanisms whereby amiloride is able to impair successful in vitro maturation.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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