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Abstract

The aim of this study was to investigate cumulus expansion, nuclear maturation and expression of connexin 43, cyclooxygenase-2 and FSH receptor transcripts in equine cumuli oophori during in vivo and in vitro maturation in the presence of equine FSH (eFSH) and precursors for hyaluronic acid synthesis. Equine cumulus-oocyte complexes (COC) were cultured in a control defined medium supplemented with eFSH (0 to 5 micrograms/ml), Fetal Calf Serum (FCS), precursors for hyaluronic acid synthesis or glutamine according to the experiments. After in vitro maturation, the cumulus expansion rate was increased with 1 microgram/ml eFSH, and was the highest with 20% FCS. It was not influenced by precursors for hyaluronic acid synthesis or glutamine. The expression of transcripts related to cumulus expansion was analyzed in equine cumulus cells before maturation, and after in vivo and in vitro maturation, by using reverse transcription-polymerase chain reaction (RT-PCR) with specific primers. Connexin 43, cyclooxygenase-2 (COX-2) and FSH receptor (FSHr) mRNA were detected in equine cumulus cells before and after maturation. Their level did not vary during in vivo or in vitro maturation and was influenced neither by FSH nor by precursors for hyaluronic acid synthesis. Results indicate that previously reported regulation of connexin 43 and COX-2 proteins during equine COC maturation may involve post-transcriptional mechanisms.

Introduction

In mammals, embryos produced in vitro, in sequential steps of oocyte maturation in vitro (IVM), conventional fertilization in vitro (IVF) or intracytoplasmic sperm injection (ICSI) and embryo culture (EC), display marked differences from their in vivo counterparts with regard to morphology, timing of development, resistance to low temperature, metabolism and gene expression [1]. Thus, their clinical applications remain sub-optimal [2-4]. Evidence has emerged to support the involvement of various

locally produced factors as co-regulators of folliculogenesis and oocyte nuclear and cytoplasmic maturation in addition to extrinsic regulation by pituitary gonadotropins and metabolic hormones [5]. Optimal expansion of the cumulus mass appears to be essential for cytoplasmic maturation [6,7]. In the bovine, the induction of cumulus expansion prior to fertilization increased the incidence of oocyte penetration [8]. In the mouse, successful fertilization was correlated with the quantity and quality of the expanded cumulus mass [9]. In the mare, cumulus expansion in oocytes retrieved from excised ovaries of slaughtered mares has been related to granulosa cell apoptosis with no relation to follicle size. Expanded oocytes issuing from apoptotic follicles show increased meiotic competence, but not increased activation rate after ICSI [10].

The process of cumulus expansion is accompanied by modifications of gap junctions, which contain transmembrane channels formed by hexamers of proteins belonging to the connexin family. Equine, bovine, ovine and mouse cumulus cells express connexin 43 proteins [11-15]. In equine, porcine and rat cumulus cells, initiation of meiotic resumption is associated with the reduction of connexin 43 protein level [11,16,17]. In the same way, during in vitro maturation of bovine cumulus-oocyte complexes, the connexin 43-positive gap junctions disappeared [12]. Prostaglandin E_2 are involved in cumulus expansion in vitro in mice [18], rats [19], and bovine [20]. The prostaglandin synthesis pathway is controled by the prostaglandin H synthase 2 also known as cyclooxygenase-2 (COX-2) [21]. In mouse, rat, bovine and equine, COX-2 protein is expressed in the granulosa cell of preovulatory follicles following hCG treatment, concomitantly with cumulus expansion [22-26]. Mice carrying a null mutation for the COX-2 exhibited abnormal expansion of the cumulus [22]. Thus, the COX-2 isoform is likely to have an important role in cumulus expansion.

In the mare, as in other species, the recent literature focuses its attention on the role of factors involved in the regulation of nuclear and cytoplasmic maturation as well as cumulus expansion, mainly those factors contained in the homologous preovulatory follicular fluid [27-29]. For example, the physiological role of epidermal growth factor (EGF) [30], insulin-like growth factor I (IGF-I) [31] or interleukin 1 (IL-1) [32] in the regulation of oocyte maturation was reported. The present study was undertaken to examine the effect of FSH in a defined medium on cumulus expansion in vitro in the equine. The predominant component in the expanded cumulus is hyaluronic acid, and hyaluronic acid synthesis is required for the volumetric expansion of the cumulus [33]. The expansion of in vitro matured mouse and sheep COCs is attenuated unless the medium contained substrates for hyaluronic acid synthesis [34]. Therefore we examined the effect on cumulus expansion in vitro of several precursors for hyaluronic acid synthesis in a defined medium. Glutamine is an initial substrates of hyaluronic acid synthesis and the addition of glutamine to the in vitro maturation medium resulted in cumulus expansion in the mouse [34], the hamster [35] and the bovine [36]. Therefore the effect of the addition of glutamine in a defined medium on cumulus expansion was tested. In order to elucidate the influence of FSH and precursors on the mechanism of equine cumulus expansion, we analyzed the expression of mRNA coding for connexin 43, COX-2 and FSH receptors during COCs maturation.

Materials and Methods

Four experiments were performed. We tested the influence of equine FSH and Fetal Calf Serum (FCS) (experiment 1), precursors for hyaluronic acid synthesis (experiment 2), eFSH concentrations (experiment 3) and glutamine (experiment 4) on nuclear maturation and cumulus expansion rates.

Cumulus-oocyte complexes recovery

Equine cumulus-oocyte complexes (COCs) were collected either by transvaginal ultrasound-guided aspiration in the standing mare (experiment 1 and 2) or from ovaries recovered from mares in a slaughterhouse during the breeding season (experiment 3 and 4).

For COCs collection by transvaginal ultrasound-guided aspiration, 60 adult cyclic pony mares in good body condition, kept indoor and fed with concentrates were used. Ovarian activity was assessed by routine rectal ultrasound scanning. An injection (i.v.) of 20 mg of crude equine gonadotropin (CEG) was performed when the largest follicle reached 33 mm in diameter to induce ovulation [37]. All follicles larger than 5 mm were punctured at the end of the follicular phase, 24 to 34 h after induction of ovulation, as previously described [38]. From follicles <30 mm, immature oocytes were collected and used for in vitro culture or immature control (see below). From preovulatory follicles (≥30 mm 34 h after induction of ovulation), mature oocytes were collected and used as in vivo mature control (see below). During follicle puncture, mares were sedated with detomidine (Domosedan, 0.6 mg/100 kg body weight (BW) i.v., Pfizer, Amboise, France) and the rectum was relaxed with propantheline bromide (20 mg/100 kg BW i.v., Sigma, Saint Quentin Fallavier, France). After follicular fluid aspiration, follicles were flushed with Phosphate Buffered Saline (PBS, Dulbecco 'A', Unipath, Dardilly, France) and heparin (50 IU/ml, LEO S.A., St-Quentin en Yvelines, France) at 37°C. Aspirated fluid from each follicle was examined individually with a stereomicroscope for COC recovery. After puncture sessions, mares received an antibiotic injection (Intramicine,

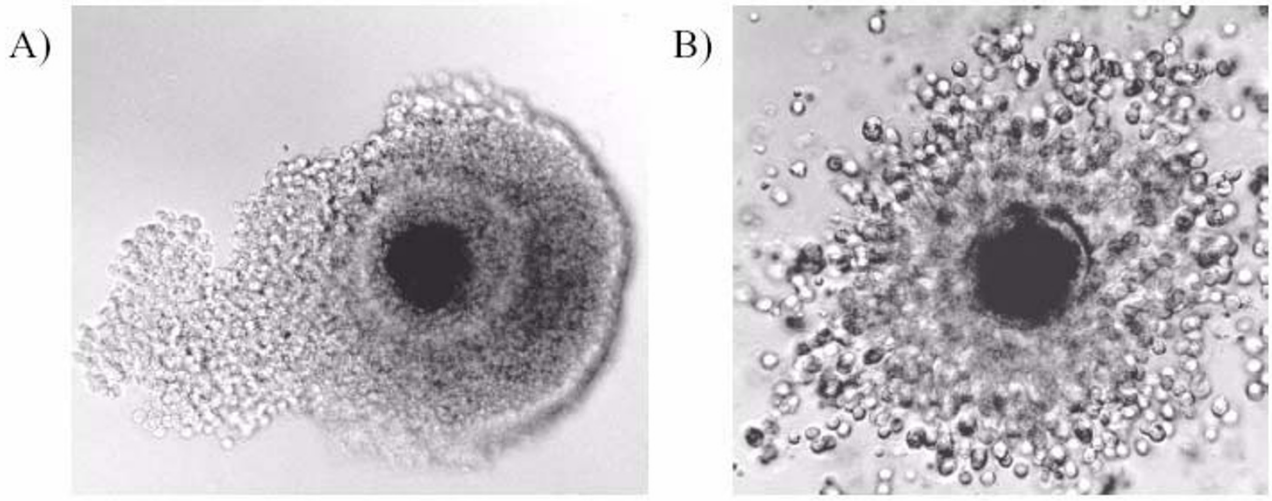

Figure 1
Compact cumulus (A): tightly attached cells surrounding the oocyte with a smooth surface over the cumulus hillock. Expanded cumulus (B): cells detached from the oocyte with matrix visible between cumulus cells.

1600000 IU penicillin/100 kg BW and 1.3 g dihydrostreptomycin/100 kg BW i.m., Rhône Mérieux, Lyon, France).

Ovaries from mares of unknown reproductive history were obtained at two local abattoirs, both located at a distance of 20 km (30 min.) from the laboratory. The ovaries were placed in physiological saline solution (9 g NaCl containing 40 mg/L gentamycin sulphate) within 30 min of slaughter and were transported to the laboratory in a thermal container (Indel "B" mod. TC18G; S. Agata F. PS – Italy) set at 30°C. Including the time taken for processing within the laboratory, the total time from slaughter to culture of oocytes was 2 to 4 hours. All follicles visible on the surface of the ovary were opened with a scalpel blade and the granulosa cell layer was scraped with a curette following the method of Hinrichs et al. [39]. Granulosa cells were flushed from the curette into individual petri dishes using tissue culture medium 199 with Earle's salts and sodium bicarbonate ($NaHCO_3$; Sigma, Milan, Italy M-2154) supplemented with 20% (v/v) heat inactivated fetal calf serum (FCS; Sigma F-4135) or 2.5% bovine serum albumin (BSA; Sigma A-4503) for oocytes destined to culture in FCS or BSA-supplemented media respectively. COCs were identified from the collected mural granulosa cells using a dissection microscope.

In vitro maturation

COCs were classified morphologically at recovery as having compact or expanded cumulus investments. Compact cumulus had tightly attached cells surrounding the oocyte with a smooth surface over the cumulus hillock (figure 1A), expanded cumulus had cells detached from the oocyte with matrix visible between cumulus cells (figure 1B). All COCs recovered were included in this study.

Since COCs were collected either in vivo in the standing mare (experiment 1 and 2) or from a slaughterhouse (experiment 3 and 4) we used two culture systems that have been shown to be appropriate for the specific requirements of those two oocyte populations.

In experiments 1 and 2, compact and expanded COCs from follicles <30 mm were washed once in maturation medium, and cultured individually in 500 μl of maturation medium in four-well dishes in a humidified atmosphere of 5% CO_2 in air at 38.5°C for 30 hours. The control maturation medium was TCM 199 with Earle's salts (Sigma, M-4530) supplemented with 5 mg/ml Bovine Serum Albumin (BSA, Sigma, A-6003), 50 ng/ml Epidermal Growth Factor (EGF, Sigma, E-4127) and antibiotics (100 IU/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml fungizone, Gibco, Eragny, France). In experiment 1, COCs were cultured in control medium, control medium with equine FSH (eFSH, 1 μg/ml, NIH, A.F. Parlow, AFP5022B n°845) or control medium without BSA with 20% inactivated FCS (Gibco, 01306290H). In experiment 2, COCs were cultured in control medium, control medium with precursors (2 mM N-acetyl-D-glucosamine (Sigma A-3286), 2 mM D-glucuronic acid (Sigma G-7906), 5 mM alpha-D-glucose-1-phosphate (Sigma, G-

1389), 1 μg/ml uridine-5'-triphosphate (Sigma, U-4630), control medium with precursors and eFSH (1 μg/ml) or control medium without BSA with 20% inactivated FCS.

In experiment 3 and 4, we used modified TCM 199 containing TCM 199 with Earle's salts and sodium bicarbonate (Sigma, Milan Italy M-2154), buffered with final 4.43 mM HEPES (Sigma H-9136) and final 33.9 mM sodium bicarbonate (Sigma S-5761) and supplemented with 0.66 mM L-glutamine (Sigma G-7513), 2 mM sodium pyruvate (Sigma P-2256), 2.92 mM calcium lactate (Serva Feinbiochem GmbH & Co Heidelberg, Germany No.29760), 50 ng/ml Epidermal Growth Factor (EGF, Sigma, E-4127) and 50 μg/ml gentamycin (Sigma G-1272). After preparation, pH was adjusted to 7.18, and the medium was filtered through 0.22-μm filters (No.5003-6, Lida Manufacturing Corp., Kenosha WI, USA). Compact and expanded COCs were washed three times in the culture medium and groups of 5 to 8 COCs with the same cumulus morphology were placed in 400 μl of medium/well of a four-well dish, covered with pre-equilibrated lightweight paraffin oil (Sigma M-3516) and cultured for 28 to 30 h at 38.5°C under 5% CO_2 in air. In experiment 3, COCs were cultured in control medium, control medium with eFSH (5 μg/ml; 1 μg/ml; 0.5 μg/ml; 0.1 μg/ml), control medium without BSA with 20% inactivated FCS. In experiment 4, COCs were cultured in control medium (modified TCM 199 + 5 mg/ml BSA + 50 ng/ml EGF), control medium with eFSH (1 μg/ml), control medium with L-glutamine (2 mM, Sigma, G-7513; the final L-glutamine concentration was 2.66 mM), control medium with eFSH plus L-glutamine or control medium without BSA with 20% inactivated FCS.

Nuclear chromatin evaluation and cumulus cells storage

As previously described, cumulus were called expanded when they had cells detached from the oocyte with matrix visible between cumulus cells. In experiments 1, 2 and 3 COCs were washed in PBS and stripped of their cumulus cells by careful aspiration in and out of a small glass pipette in PBS to store the cumulus cells without culture medium or additives. The PBS solution containing the cumulus cells was centrifuged (13000 g, 1 minute) in order to obtain a cells pellet. The supernatant was carefully removed, pellets were frozen in liquid nitrogen and stored at -80°C until use. In experiment 4 cumulus cells were removed by incubation in TCM 199 with 20% FCS containing 80 IU/ml hyaluronidase (Sigma H-3506) and by careful aspiration in and out of finely drawn glass pipettes. Cumulus cells were not stored.

COCs that were classified at recovery as having compact or expanded cumulus were assessed for oocyte morphology by evaluating the uniformity of the ooplasm, the perivitelline space, the presence of an intact zona pellucida and oolemma and the first polar body (PB) extruded. To evaluate nuclear chromatin, totally denuded oocytes were stained with 1 μg/ml bisbenzimide fluorescent dye (Hoechst 33342, Sigma) in PBS and observed in a drop on a slide with an epifluorescence microscope (experiments 1 and 2) or fixed in 3.8% buffered formaldeyde solution (J.T. Baker; N° 7385), stained with 2.5 μg/ml Hoechst 33258 (Sigma B-1155) in 3:1 (v/v) glycerol/PBS and observed under an E-600 Nikon fluorescent microscope (experiments 3 and 4). Nuclear chromatin status was classified as follows: germinal vesicle (GV); metaphase to telophase I (MI) and complete maturation at metaphase II (MII) with the first polar body extruded. Oocytes showing either multipolar meiotic spindle or irregular chromatin clumps or no chromatin were considered to be abnormal. Oocytes with fragmented or shrunkled cytoplasm were classified as degenerated.

In order to obtain an in vivo mature control and an immature control for further analysis, expanded COCs collected from preovulatory follicles (≥30 mm) and compact COCs from small follicles (<30 mm) were collected by in vivo transvaginal follicular punctures as in experiment 1. For the in vivo mature control, expanded COCs collected from preovulatory follicles were examined at collection as in experiment 1. Only cumulus cells associated with oocytes that reached metaphase II after in vivo maturation were stored. For the immature control, compact COCs from small follicles were examined at collection as in experiment 1. Only cumulus cells associated with oocytes that were at the germinal vesicle stage were stored.

Extraction of total RNA and RT-PCR

Total RNA was extracted from equine cumulus cells using the TriPure Isolation Reagent Kit (Boehringer Mannheim) according to the manufacturer's recommendations. Briefly, pools of 2 to 9 cumulus cells pellets were mixed with 100 μl of reagent with 1 μg of glycogen (Boehringer Mannheim) as a carrier and added to 60 μl chloroform. After centrifugation, RNA in the upper aqueous phase was recovered, precipitated with isopropanol, washed with ethanol and dried. After extraction, RNA pellets were resuspended in 4 μl of water and stored at -20°C.

Prior to the reverse transcription (RT) reaction, the RNA samples were incubated for 10 min at 70°C and chilled on ice. The reverse transcription reaction was conducted in a final volume of 10 μl, containing 4 μl of extracted RNA, 2 μM oligo$(dT)_{12\text{-}18}$ (Amersham Pharmacia, Orsay, France), 1X reverse transcriptase buffer (Promega, Charbonnière, France), 2.5 mM $MgCl_2$ (Promega), 1.25 mM of each dNTP (Promega), 20 IU of recombinant ribonuclease inhibitor (RNasin, Promega), and 100 IU of Moloney murine leukemia virus reverse transcriptase (M-MLV, Promega). The reaction was performed for 60 min at

37°C, then reverse transcriptase was heat-inactivated for 5 min at 95°C.

PCR amplification of FSH receptor, connexin 43, cyclooxygenase 2 and glyceraldehyde-3-phosphate deshydrogenase (GAPDH) cDNA were performed with 4 μl of RT product, in a final volume of 25 μl containing 1X Taq DNA polymerase buffer, 1.5 mM of $MgCl_2$ (Promega), 0.2 μM of each dNTP (Promega), 0.5 μM of each sense and antisense primer (Eurogentec, Nantes, France), and 1 IU of Taq DNA polymerase (Promega). PCR reactions were performed in a thermal cycler (geneamp PCR system 9700, Perkin Elmer, USA). The initial denaturation was conducted at 94°C for 3 min, followed by 40 cycles of 30 sec at 94°C, 30 sec at primer hybridization temperature (60°C), and 45 sec at 72°C. Final elongation was performed at 72°C for 3 min. The RT-PCR products were subjected to electrophoresis on a 2% agarose gel containing 0.25 μg/ml ethidium bromide. Signals were visualized by ultraviolet illumination and quantified using image analysis software (ImageMaster; Molecular Dynamics). To obtain the relative levels of FSH receptor, connexin 43 and cyclooxygenase 2 mRNA, we calculated the ratio of the intensity of the corresponding signal to the intensity of the signal for GAPDH mRNA. PCR reaction was done separately for each amplification.

Primers for equine GAPDH were based on the sequence of the equine GAPDH cDNA published in Genbank (accession number AF157626, [40]). The upstream primer (5'-GTTTGTGATGGGCGTGAACC-3') and the downstream primer (5'-TTGGCAGCACCAGTAGAAGC-3') predict a 255 bp DNA fragment. Primers for equine FSH receptor (FSHR) were based on the sequence of the equine FSHR cDNA published in Genbank (accession number S70150, [41]). The upstream primer (5'-TCTTTGGCATCAGCACCTAC-3') and the downstream primer (5'-AGAAATCCCTGCGGAAGTTC-3') predict a 399 bp DNA fragment. Primers for equine connexin 43 were based on the sequence of the equine connexin 43 cDNA published in Genbank (accession number AF042352). The upstream primer (5'-TGCCTTGAACGTCATCGAAC-3') and the downstream primer (5'-TTGGTGAAGAGCAGCCATTG-3') predict a 161 bp DNA fragment. Primers for equine cyclooxygenase 2 (COX-2) were based on the sequence of the equine COX-2 cDNA published in Genbank (accession number AB041771). The upstream primer (5'-ATACCAAAACCGCATTGCCG-3') and the downstream primer (5'-TCTAACTCCGCAGCCATTTC-3') predict a 360 bp DNA fragment.

As negative controls, tubes without RNA or reverse transcriptase were used for RT-PCR. Omitting RNA or reverse transcriptase did not generate any amplified fragments (data not shown).

Statististical analysis

The Chi-square test was used to compare oocyte maturation rates and cumulus expansion rates. The non parametric Kruskal-Wallis test was performed using StatXact 4 software (CYTEL, Cambridge, MA) in order to compare means of gene expression signals.

Results

Oocyte recovery

In experiment 1, 158 follicles from 5 to 30 mm were punctured in vivo in 19 mares, and 71 COCs were recovered. Averages of 0.45 COCs per follicle and 1.9 COCs per ovary were obtained. In experiment 2, 315 follicles from 5 to 30 mm were punctured in vivo in 35 mares, and 126 COCs were recovered. Averages of 0.4 COCs per follicle and 1.8 COCs per ovary were obtained. In experiment 3, from the ovaries of 25 slaughtered mares, 174 oocytes were recovered for a recovery rate of 3.41 oocytes per ovary. In experiment 4, from the ovaries of 40 slaughtered mares, 360 oocytes were recovered for a recovery rate of 4.44 oocytes per ovary.

All COCs were included in the analysis in that none of them were lost or damaged during handling before and after culture, and all of them had enough cumulus cells for molecular analysis.

Nuclear maturation and cumulus expansion

In experiment 1, COCs were cultured in control medium, control medium with eFSH or control medium without BSA with FCS. The nuclear maturation rates were not significantly different between the 3 media (table 1). The addition of eFSH did not significantly increase the cumulus expansion rate (48% vs 42% in the control medium). The cumulus expansion rate was significantly higher in the presence of FCS (83%) when compared to the control medium (42%, $p < 5\%$) (table 1).

In experiment 2, COCs were cultured in control medium, control medium with precursors, control medium with precursors and eFSH or control medium without BSA with FCS. The nuclear maturation rates were not significantly different between the 4 media (table 2). The addition of precursors or precursors and eFSH did not significantly increase the cumulus expansion rate (48% or 57% vs 53% in the control medium). The cumulus expansion rate was significantly higher in the presence of FCS (84%) when compared to the control medium (53%, $p < 5\%$) (table 2).

In experiment 3, COCs were cultured in control medium, control medium supplemented with different concentrations of eFSH, or control medium without BSA with FCS. The addition of FSH or FCS did not influence the nuclear maturation rate when compared to the control

Table 1: Effect of eFSH and FCS on nuclear maturation and cumulus expansion.

Maturation medium	Nuclear maturation rate % (n° of oocytes)	Cumulus expansion rate % (n° of oocytes)
Control	70% (21) [a]	42% (21) [a]
Control + 1 μg/ml eFSH	62% (21) [a]	48% (21) [a]
Control without BSA with 20% FCS	74% (27) [a]	83% (29) [b]

[a,b] Values with different superscripts are significantly different ($p < 0.05$).

Table 2: Effect of precursors for hyaluronic acid synthesis on nuclear maturation and cumulus expansion.

Maturation medium	Nuclear maturation rate % (n° of oocytes)	Cumulus expansion rate % (n° of oocytes)
Control	56% (34) [a]	53% (32) [a]
Control + precursors	61% (31) [a]	48% (31) [a]
Control + precursors + 1 μg/ml eFSH	59% (30) [a]	57% (29) [a]
Control without BSA with 20% FCS	65% (31) [a]	84% (31) [b]

[a,b] Values with different superscripts are significantly different ($p < 0.05$).

Table 3: Effect of eFSH concentration on nuclear maturation and cumulus expansion.

Maturation medium	Nuclear maturation rate % (n° of oocytes)	Cumulus expansion rate % (n° of oocytes)
Control	50% (28) [a]	33% (15) [a]
Control + 0.1 μg/ml eFSH	27% (26) [a]	36% (14) [a]
Control + 0.5 μg/ml eFSH	37% (27) [a]	27% (15) [a]
Control + 1 μg/ml eFSH	37% (27) [a]	47% (15) [a]
Control + 5 μg/ml eFSH	50% (28) [a]	43% (14) [a]
Control without BSA with 20% FCS	50% (26) [a]	100% (14) [b]

[a,b] Values with different superscripts are significantly different ($p < 0.05$).

medium, whatever the FSH concentration (table 3). The addition of eFSH did not significantly influence the cumulus expansion rate, whatever the FSH concentration (27 to 47% vs 33% in the control medium). The cumulus expansion rate was significantly higher in the presence of FCS (100%) when compared to the control medium (table 3).

In experiment 4, COCs were cultured in control medium, control medium with eFSH, control medium with L-glutamine, control medium with eFSH plus L-glutamine or control medium without BSA with FCS. The addition of FSH, glutamine or FCS did not influence the nuclear maturation rate when compared to the control medium (table 4). The cumulus expansion rate was significantly higher in the presence of eFSH (60%) or FCS (94%) when compared to the control medium (32%, $p < 5\%$). The cumulus expansion rate was not improved by the presence of glutamine alone (41%) or glutamine + eFSH (51%) when compared to the control medium or the control medium with eFSH (table 4).

FSH receptor, connexin 43 and cyclooxygenase 2 expression in cumulus cells

In experiment 1, we used 2 pools of 4 cumulus cells pellets from control medium, 2 pools of 4 pellets from medium containing FSH, 2 pools of 4 pellets from medium containing FCS, as well as 2 pools of 2 pellets from in vivo mature control, and 2 pools of 3 pellets from immature control. Amplification of cDNA with equine connexin 43 specific primers resulted in one PCR product with the expected size of 161 bp (figure 2A). Amplification of cDNA with equine COX2 specific primers resulted in one PCR product with the expected size of 360 bp (figure 2B). The connexin 43 and COX2 products were detected in all cumulus cells whether they were from immature control, from in vivo mature control, or from in vitro maturation. No PCR products were detected when a water control was

Table 4: Effect of glutamine on nuclear maturation and cumulus expansion.

Maturation medium	Nuclear maturation rate % (n° of oocytes)	Cumulus expansion rate % (n° of oocytes)
Control	50% (67) [a,b]	32% (67) [a]
Control + 1 μg/ml eFSH	34% (86) [a]	60% (86) [b]
Control + 2 mM glutamine	36% (59) [a,b]	41% (59) [a,b]
Control + 2 mM glutamine + 1 μg/ml eFSH	34% (80) [a]	51% (80) [a,b]
Control without BSA with 20% FCS	53% (68) [b]	94% (68) [c]

[a,b,c] Values with different superscripts are significantly different ($p < 0.05$).

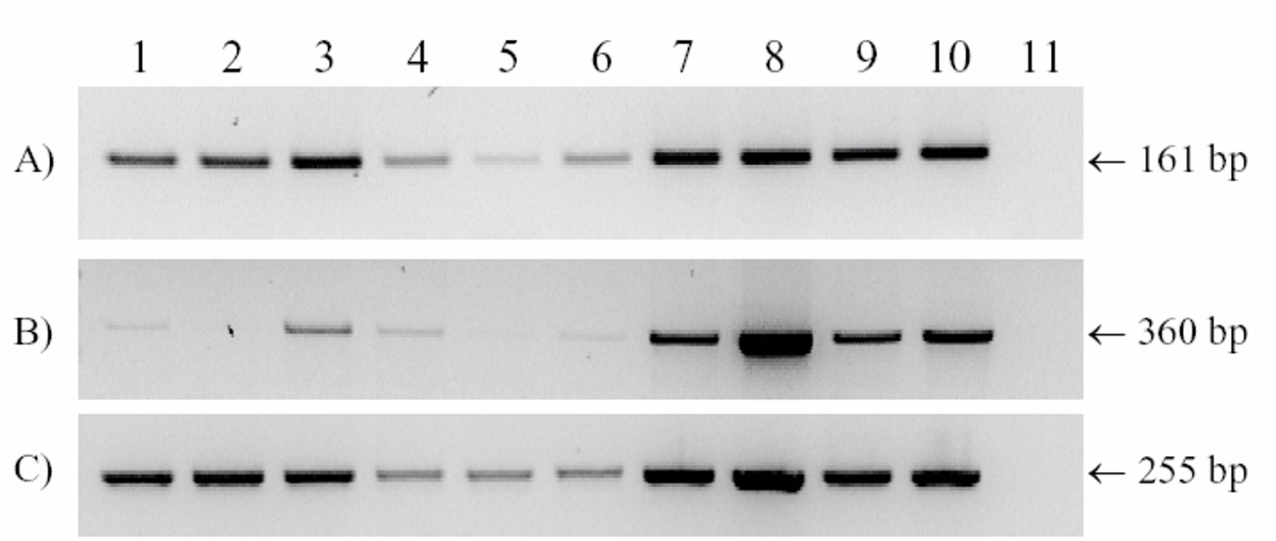

Figure 2
Connexin 43 (A), cyclooxygenase 2 (B) and GAPDH (C) mRNA expression in equine cumulus cells as detected by RT-PCR. Lanes 1 and 2: 2 pools of 4 cumulus cells pellets from medium containing FCS, lanes 3 and 4: 2 pools of 4 pellets from control medium, lanes 5 and 6: 2 pools of 4 pellets from medium containing FSH, lanes 7 and 8: 2 pools of 2 pellets from in vivo mature control, lanes 9 and 10: 2 pools of 3 pellets from immature control, lane 11: H_2O.

used as template for the PCR. As a control for RNA isolation and the production of cDNA, all samples were amplified with equine GAPDH specific primers. A strong GAPDH product of expected size (255 bp) was detected in all samples (figure 2C). PCR amplification of connexin 43, COX2 and GAPDH was repeated twice, and we took the average of the 2 quantifications. The relative amount of connexin 43 and cyclooxygenase 2 mRNA was normalized to a constant amount of GAPDH mRNA. No obvious differences were observed for the expression of connexin 43 or COX2 between the different culture media, the in vivo mature control and the immature control.

In experiment 2, we used 3 pools of 2 cumulus cells pellets from control medium, 3 pools of 2 pellets from medium containing precursors, 3 pools of 2 pellets from medium containing precursors and FSH, and 3 pools of 2 pellets from medium containing FCS. Amplification of cDNA with equine connexin 43 and COX2 specific primers resulted in PCR products with the expected size of 161 bp and 360 bp respectively (figure 3A and 3B). The connexin 43 and COX2 products were detected in all cumulus cells whatever the in vitro culture medium (figure 3). No PCR products were detected when a water control was used as template for the PCR. PCR amplification of connexin 43, COX2 and GAPDH was repeated twice, and we took the average of the 2 quantifications. No obvious differences

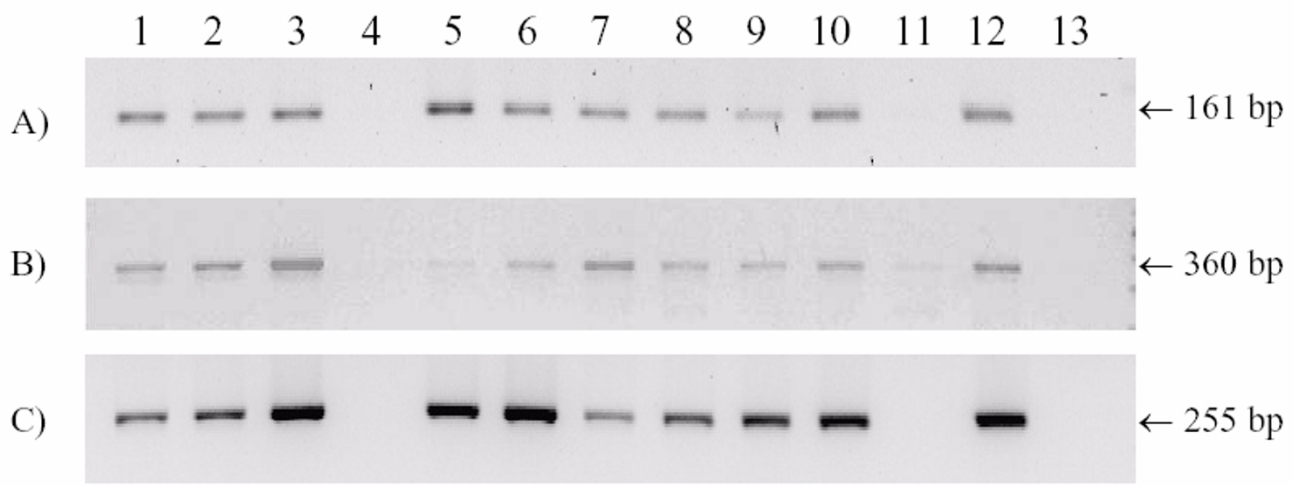

Figure 3
Connexin 43 (A), cyclooxygenase 2 (B) and GAPDH (C) mRNA expression in equine cumulus cells as detected by RT-PCR. Lanes 1, 2 and 3: 3 pools of 2 cumulus cells pellets from control medium, lanes 4, 5 and 6: 3 pools of 2 pellets from medium containing precursors, lanes 7, 8 and 9: 3 pools of 2 pellets from medium containing precursors and FSH, lanes 10, 11 and 12: 3 pools of 2 pellets from medium containing FCS, lane 13: H_2O.

were observed for the expression of connexin 43 or COX2 normalized to GAPDH between the different culture media.

In experiment 3, COCs that were classified at recovery as having compact or expanded cumulus were analyzed separately. We used 3 pools of 5 compact cumulus and 3 pools of 3 to 6 expanded cumulus from control medium, 3 pools of 5 compact cumulus and 3 pools of 3 to 6 expanded cumulus from medium containing FCS, 3 pools of 3 to 8 compact cumulus and 3 pools of 3 to 9 expanded cumulus for each FSH concentration. Amplification of cDNA with equine connexin 43 specific primers resulted in one PCR product with the expected size of 161 bp (figure 4A). Amplification of cDNA with equine FSH receptor specific primers resulted in one PCR product with the expected size of 399 bp (figure 4B). The connexin 43 and FSH receptor products were detected in all cumulus cells whatever the in vitro culture medium (figure 4). No PCR products were detected when a water control was used as template for the PCR. PCR amplification of connexin 43, FSH receptor and GAPDH was repeated twice, and we took the average of the 2 quantifications. No obvious differences were observed for the expression of connexin 43 or FSH receptor normalized to GAPDH between the different culture media or between compact and expanded cumulus.

Discussion

The aim of this study was to improve in vitro cumulus expansion in the equine COCs, since optimal expansion of the cumulus mass is essential for cytoplasmic maturation. We examined the effect of FSH and precursors for hyaluronic acid synthesis. In order to elucidate the mechanism of equine cumulus expansion, we analyzed the expression of connexin 43, COX-2 and FSH receptors mRNA during COCs maturation.

FSH induces cumulus expansion in several mammalian species. In mouse COCs, rat, ovine and porcine FSH was able to stimulate cumulus expansion [42,43]. In bovine COCs, cumulus expansion was induced by bovine, porcine and human FSH in defined culture medium, but no significant difference existed in the percentage of oocytes reaching metaphase II [44,45]. In porcine COCs, ovine FSH had no effect on the proportion of oocytes undergoing GVBD but it stimulated cumulus expansion [46-48]. To our knowledge, only one paper reports the influence of FSH on equine COCs expansion [49]. The authors showed that bovine FSH did not significantly change the number of metaphase II oocytes but increased the cumulus expansion. However, the oocytes in this experiment were incubated in a medium containing estrus mare serum. Estrus mare serum may contain FSH, or interact with exogenous FSH. To understand the influence of hormones during IVM, all products with undefined components should be eliminated from culture condi-

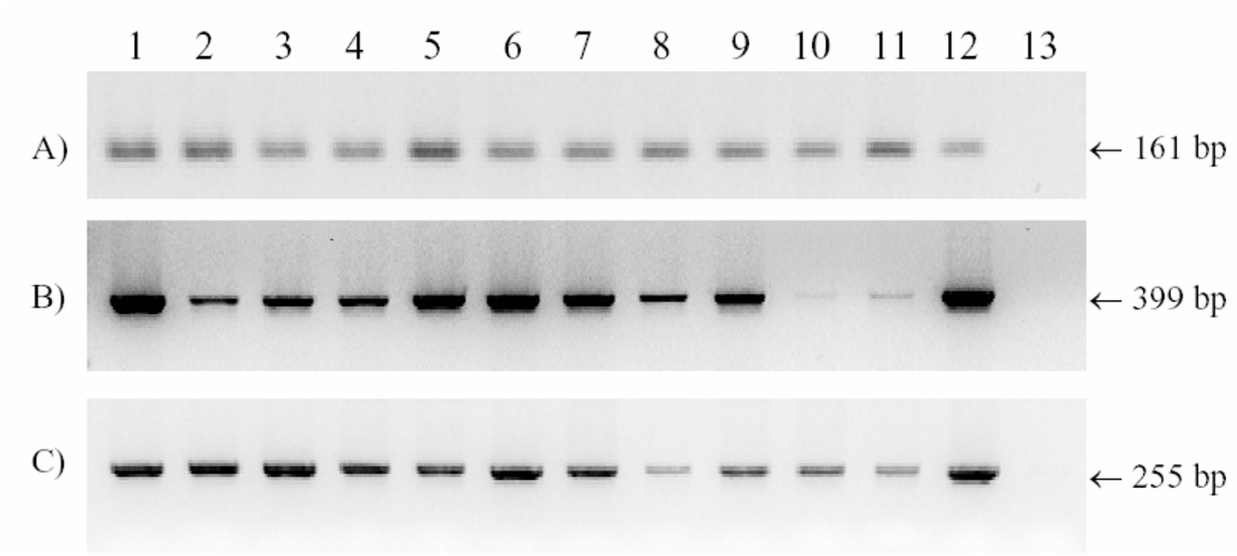

Figure 4
Connexin 43 (A), FSH receptor (B) and GAPDH (C) mRNA expression in equine cumulus cells as detected by RT-PCR. Lane 1: 5 compact cumulus cells from medium containing FCS, lane 2: 5 compact cumulus cells from control medium, lane 3: 3 compact cumulus cells from medium containing 5 μg/ml FSH, lane 4: 4 compact cumulus cells from medium containing 1 μg/ml FSH, lane 5: 3 compact cumulus cells from medium containing 0.5 μg/ml FSH, lane 6: 6 compact cumulus cells from medium containing 0.1 μg/ml FSH, lane 7: 6 expanded cumulus cells from medium containing FCS, lane 8: 3 expanded cumulus cells from control medium, lane 9: 3 expanded cumulus cells from medium containing 5 μg/ml FSH, lane 10: 3 expanded cumulus cells from medium containing 1 μg/ml FSH, lane 11: 5 expanded cumulus cells from medium containing 0.5 μg/ml FSH, lane 12: 9 expanded cumulus cells from medium containing 0.1 μg/ml FSH, lane 13: H_2O.

tions. That is why, in our lab, the evaluation of culture medium for equine oocytes has progressed toward more defined conditions. BSA-supplemented media are widely used as defined media in labs working with equine oocytes [50,51]. In experiments 1 and 3, equine FSH did not influence cumulus expansion in defined culture medium. However, since the cumulus expansion rate was higher, though the difference was not significant, with 1 μg/ml eFSH than without eFSH, we repeated the experiment with a high number of oocytes. In experiment 4, 1 μg/ml eFSH significantly increased the cumulus expansion rate. Those results are consistent with data from other mammalian species. The FSH concentration which is efficient in vitro in our study (1 μg/ml) is in the range of the efficient concentrations in the other mammals. Moreover, as in the other species, eFSH has no effect on the proportion of equine oocytes reaching metaphase II.

Information about the mechanism of FSH action on the cumulus cells is limited. In bovine and porcine, a direct role for FSH in cumulus expansion has been supported by the demonstration of receptors for FSH: RT-PCR and in situ hybridization experiments revealed that mRNA for FSH receptor was present in bovine [32,52] and porcine [53] cumulus cells. In our study, the influence of FSH on equine cumulus expansion was supported by the detection of mRNA for the FSH receptor in equine cumulus cells from immature and mature COCs. Expression of FSH receptor transcripts was previously reported in equine cumulus cells from immature COCs [54]. But to our knowledge, the expression of mRNA for the FSH receptor during in vivo and in vitro maturation has been reported neither in the equine, nor in the other domestic mammals. Together with previous studies that used ligand binding to demonstrate that specific FSH receptors are present within equine follicles [55], our finding confirms the production and expression of the FSH receptor in equine follicles.

In the equine, the volumetric expansion of in vitro matured COCs is generaly lower than that observed to occur in vivo [56,57]. The synthesis of the hyaluronic acid matrix in cumulus mass plays a significant role in the volumetric expansion of the cumulus [33]. It seemed possible that the medium used in the culture of COCs lacks sufficient concentrations of substrates to mimic the magnitude of hyaluronic acid synthesis and cumulus expansion normally observed in vivo. When the synthesis of hyaluronic acid was supported by the addition of glucosamine, N-acetylglucosamine or UDP-N-acetylglu-

cosamine, the degree of expansion in mouse COCs nearly approached that observed to occur in vivo [34]. In experiment 2 we hypothesised that greater spatial expansion of equine COCs occurred in culture in the presence of substrates of hyaluronic acid synthesis. The maturation medium that we used in this study contained several substrates described in the pathway of hyaluronic acid synthesis [34], but no N-acetylglucosamine, glucuronic acid, glucose-1-phosphate, or UTP (uridine triphosphate). Those substrates may be limiting factors for hyaluronic acid synthesis and so for cumulus expansion. In experiment 2, we tested the influence on cumulus expansion of a defined culture medium supplemented with those substrates. Unfortunately, the addition of those substrates in the culture medium of equine COCs did not increase the cumulus expansion rate. The limiting factor for cumulus expansion does not seem to be N-acetylglucosamine, glucuronic acid, glucose-1-phosphate, or UTP.

Glutamine is a metabolic precursor for hexosamine synthesis and its inclusion in culture medium has been reported to improve cumulus expansion. In mouse COCs, maximal expansion was achieved by addition of 3 mM glutamine [34]. In hamster COCs, cumulus expansion occurred in the presence of 0.2 mM glutamine [35]. In bovine COCs, the addition of 2 mM glutamine stimulated expansion and improved cleavage and blastocyst rates [36]. Experiment 4 was designed to evaluate the effect of glutamine during in vitro culture on equine cumulus expansion. In our study, the control medium contained 0.66 mM L-glutamine. Since the glutamine concentration that is efficient in the mouse and the bovine ranges between 2 and 3 mM, we supplemented the culture medium with 2 mM L-glutamine to reach a final concentration of 2.66 mM. The addition of 2 mM glutamine to our control medium did not increase the cumulus expansion rate.

The process of cumulus expansion and the synthesis of hyaluronic acid can be induced in vitro by FSH. Moreover, the synthesis of the hyaluronic acid matrix require substrates of hyaluronic acid. Therefore, to achieve expansion comparable with that observed in vivo, the medium should contain sufficient hyaluronic acid substrates in addition to FSH. We tested the influence on cumulus expansion of the addition of FSH and precursors for hyaluronic acid (experiment 2) and FSH and glutamine (experiment 4). However, none of these additives was efficient to improve the cumulus expansion rate.

To ascertain that the COCs were able to expand in our conditions, we used a medium supplemented with FCS as a positive control. Actually, FCS has been widely used in the culture of COCs to induce cumulus expansion. Rat, mouse and sheep COCs expanded in media containing FCS [34]. Porcine COCs that matured in the serum of pig or cow showed an expansion of the surrounding cumulus cell matrices [58]. Bovine COCs showed high cumulus expansion with either 5 or 10% FCS [59]. In fact, serum components allow retention of synthesized hyaluronic acid within the COC [60,61]. In the equine, use of mare serum led to 80% cumulus expansion, and the highest degree of cumulus cell expansion (100%) was achieved with FCS [49]. In our study, the cumulus expansion rate in culture media supplemented with FCS ranged from 83 to 100%. Those results are consistent with previous results in the mare, and confirm that the COCs were competent for expansion.

The cumulus cells are functionally interconnected by gap junctions. The fundamental unit of the gap junction is the connexon, which is a hexamer of proteins called connexins. We have begun to characterize the connexins that contribute to gap junctions in the equine COC, and we reported the expression of connexins 43 protein in the equine cumulus cells [11]. As previously observed in rat, porcine, and bovine cumulus cells [12,16,17], initiation of meiotic resumption is associated with the reduction of connexin 43 protein level in the equine COC [11]. In this study, we showed that the connexin 43 mRNA level did not vary during in vitro or in vivo maturation. To our knowledge, the studies about the expression of mRNA coding for connexin 43 in the follicle are scarce, and no study report the expression of connexin 43 mRNA in cumulus cells. Our study showed that the reduction of connexin 43 protein level during COC maturation is not due to a decrease in the mRNA level. One can hypothesize that post-transcriptional events are involved in this process. On the contrary, in the rat, connexin 43 protein in the preovulatory follicles decreased after the LH surge, and concomitantly the level of connexin 43 mRNA expressed in the granulosa cells dropped [62]. The regulation of the connexin 43 protein level may involve different mechanisms according to the species (rat vs equine) and/or the cell type (cumulus vs granulosa). Further studies are required to elucidate these mechanisms. During in vitro maturation of equine COCs, neither FSH nor precursors for hyaluronic acid synthesis influenced the expression of connexin 43 mRNA in cumulus cells. Using a rat granulosa cells line expressing the FSH receptor and the connexine 43 gene, Sommersberg et al [63] showed that treatment with FSH increased connexin 43 mRNA level. Those results reinforce the hypothesis that the regulation of the connexin 43 expression may involve different mechanisms according to the species and the cell type.

A role for prostaglandin E_2 (PGE_2) in cumulus expansion has been shown in mice [18], rats [19] and bovine [20]. Prostaglandins are generated by the cyclooxygenase (COX) pathway. COX is the rate-limiting enzyme for the

conversion of arachidonic acid into PGH2, the precursor for various prostaglandins. Two isoforms have been identified. COX-1 is a constitutive enzyme, that is expressed in many tissues to ensure the synthesis of prostaglandins for the so-called housekeeping functions [64]. COX-2 is an inducible enzyme that appears as an early responsive gene triggered by a wide variety of factors. In granulosa cells of preovulatory follicles, COX-2 protein expression was induced by hCG treatment [mouse: [22,23]; rat: [24]; bovine: [25]; equine: [26]]. In cumulus cells of mouse COCs, expression of COX-2 mRNA and protein was also induced by hCG treatment [65,22,23]. In cumulus cells of bovine COCs, no COX-2 labelling was detected in immature COCs, whereas a strong labelling was observed in in vivo matured COCs [66]. Until now, the expression pattern of COX-2 in equine COCs was unknown. We demonstrated in the present study that COX-2 was expressed by equine cumulus cells in immature COCs as well as in COCs after in vivo and in vitro maturation. The expression level of COX-2 mRNA did not vary between immature COCs and in vivo matured COCs. Since the expression of COX-2 protein in equine granulosa cells increased during in vivo preovulatory maturation [26] and the expression of COX-2 transcripts did not vary (this study), the regulation of COX-2 in the equine follicle may involve post-transcriptional events. However, the post-transcriptional modifications can be only hypothesized and remain to be verified. In our conditions of in vitro culture, the expression level of COX-2 mRNA did not vary between immature COCs and in vitro matured COCs. Moreover, neither FSH nor precursors for hyaluronic acid synthesis influenced the expression of COX-2 mRNA in cumulus cells. In bovine in vitro cultured COCs, the level of COX-2 transcript and protein within cumulus cells was undetectable or low at 0 h of culture, it remained unchanged after 24 h in culture in TCM199 alone or in TCM199 supplemented with FSH and LH, whereas addition of EGF in maturation medium caused a marked increase in COX-2 mRNA and protein quantity [20,66,67]. In mouse in vitro cultured COCs, levels of COX-2 mRNA in cumulus cells were low after 4 h in culture in TCM199 plus BSA supplemented or not with LH, whereas addition of FSH initiated high levels of COX-2 mRNA expression [67]. Thus, the level of COX-2 expression in cumulus cells was affected by conditions of in vitro culture. Since the culture media used in our and previous studies were different, comparisons between species are not reliable, however the influence of different additives in the culture medium on COX-2 expression may be species-specific.

Conclusions

In conclusion, we observed for the first time the expression of FSH receptors, connexin 43 and COX-2 transcripts in equine cumulus cells during in vivo and in vitro maturation. We showed that FSH increased equine cumulus expansion in defined culture medium, but precursors for hyaluronic acid synthesis did not.

Authors' contributions

GG, MED and PM conceived and designed the study. GG carried out and coordinated the experimental procedures in experiment 1 and 2. MED carried out and coordinated the experimental procedures in experiment 3 and 4. MC and AM worked in ex vivo oocyte collection and nuclear stage evaluation. GG and NG performed the PCR analyses. FM and GA performed the in vitro cultures and nuclear stage evaluations of in vitro recovered oocytes. FM and GA also participates in ex vivo oocyte collection and nuclear stage evaluation. GG and MED performed the statistical analysis and wrote the manuscript. All the Authors read and approved the final manuscript.

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