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Short title: Non-invasive evaluation of equine oocytes

Abstract

The purpose of this study was to observe *in vitro* matured equine oocytes using a polarized light microscope (PLM) to compare the subjective morphological evaluation obtained using a classic light microscope (LM) with an objective computerized evaluation.

Equine *cumulus*-oocyte complexes (COCs, n=922) were subjected to different *in vitro* maturation times (24, 36, or 45 h), but only 36-h matured oocytes were analyzed using PLM.

The 36-h matured oocytes that reached maturity were parthenogenetically activated to evaluate quality and meiotic competence. Average maturation percentages per session in groups 1, 2, and 3 (24-, 36- and 45-h matured oocytes, respectively) were 29.31±13.85%, 47.01±9.90%, and 36.62±5.28%, whereas the average percentages of immature oocytes per session was

28.78±20.17%, 7.83±5.51%, and 22.36±8.39%, respectively. The zona pellucida (ZP) birefringent properties were estimated and correlated with activation outcome. ZP thickness and retardance of the inner layer of the zona pellucida (IL-ZP) were significantly increased in immature oocytes compared with mature oocytes ($p<0.001$ and $p<0.01$, respectively). The comparison between parthenogenetically activated and non-activated oocytes showed a significant increase in the area and thickness of the IL-ZP in parthenogenetically activated oocytes ($p<0.01$). These results show that the 36-h IVM protocol allowed equine oocytes to reach maturity, and PLM observation of ZP can be used to distinguish mature and immature oocytes as well as activated and non-activated oocytes.

1. Introduction

In vitro maturation (IVM) of oocytes is a common technique used for years in assisted reproduction programs in veterinary medicine. It allows the successful maturation of oocytes recovered at any developmental stage by employing specific culture media and conditions. During this process, gametes undergo structural and biological modifications before proceeding in their maturity (Carneiro *et al*. 2002).

There is a wide degree of variability in the maturation competence of equine oocytes (Hinrichs 2010), and only a restricted number of cells can reach the maturation stage suitable for subsequent fertilization. Identifying predictive markers of *in vitro* developmental competence could achieve higher performance percentages in assisted reproduction techniques. Analysis of *cumulus oophorus*, cytoplasmic and nuclear aspects, polar body presence, vital staining, and parthenogenetic activation are suitable for these purposes. Some authors have stated that some morphological parameters of oocytes and *cumulus oophorus* are useful elements for evaluating gamete quality, including the expansion and integrity grade of *cumulus oophorus*, the presence of polar bodies, the absence of ZP abnormalities, and the absence of cytoplasmic fragments in the perivitelline space (Dell'Aquila *et al*. 1997a, Galli *et al*. 2007, Caamaño *et al*. 2010).

Other techniques require either fixation and/or fluorescence staining and confocal microscopy to analyze the spindle structure following immunostaining of chromatin and/or tubulin and electron microscopy techniques (Tharasanit *et al*. 2006; Siddiqui *et al*. 2009). These methods have various disadvantages including their invasiveness, which renders the oocytes unusable after evaluation. Other methods such as brilliant cresyl blue staining are not useful for predicting equine oocyte competence prior to IVM (Pereira *et al*. 2014). Moreover, the static nature of classical microscopy images impairs the dynamic evaluation of oocyte behavior (Caamaño *et al*. 2010).

In this context, PLM, a technique normally used for characterization of human oocytes in assisted fertilization treatments, offers dynamic evaluation of oocyte behavior and could be useful for equine assisted reproduction.

The purpose of this study was to observe equine oocytes using PLM to compare the subjective morphological evaluation obtained using a classic light microscope (LM) with an objective computerized evaluation applied in human *in vitro* fertilization (IVF), which is one of the method adopted for oocyte quality evaluation.

The literature describes different maturation times for equine oocytes based on *cumulus* morphology (culture is performed for 24-30 h for expanded oocytes and 30-36 h for compact oocytes) (Hinrichs *et al*. 2005). According to previous work, the collected *cumulus*-oocyte complexes (COCs) were classified based on *cumulus* morphology and subjected to 24-, 36- or 45-h IVM. Since 36-h matured oocytes exhibited better maturity and viability, and due to the lack of available facilities at the Obstetric Gynecological Hospital Sant'Anna in Turin, only the 36-h oocytes were observed under PLM to identify morphological signs of successful maturation.

2. Materials and Methods

2.1. Ovary collection and dissection

The ovaries were collected over a 14-month period at a local abattoir immediately after evisceration of 104 mares with unknown reproductive history and transported at 35-37°C in a sterile

physiological saline solution (SPSS) to the laboratory within 4-5 h to ensure the best standardized conditions (Gambini *et al*. 2014). Immature or abnormal gonads (n=10) were excluded from the study. Upon arrival, ovaries were dissected from the surrounding connective tissue, rinsed with a 10% Virkon® solution, and maintained in SPSS at a temperature of 35-37°C.

A laminar flow hood, sterile surgical instruments, and sterile gloves were used for follicle dissection. First, the *tunica albuginea* and interstitial tissue were removed to expose the follicular wall. Each follicle was sliced using a scalpel blade, and the antral surface of the follicular wall was scraped using a Volkmann spoon to ensure the separation of the COCs.

The follicular contents were collected in 50-mL Petri dishes and mixed with dissection medium (DM), which was composed of M199 (15.06 g/L) supplemented with penicillin (0.07 g/L), streptomycin (0.07 g/L), heparin (0.01 g/L), BSA (bovine serum albumin, 1 g/L), NaOH (0.52 g/L), and $NaHCO_3$ (0.34 g/L) at 38°C; the pH was adjusted to 7.2-7.4. The medium was placed in a humidified incubator at 38.5°C with 5% CO_2. Using this same medium, each dissected follicle was washed using a 20-mL syringe with a 21-G needle. The number of dissected follicles was recorded for each Petri dish to quantify the gamete recovery rate (Table 1). The ovaries (n=187) were processed (21 ovaries were eliminated), and a total of 922 oocytes were collected from 1331 dissected follicles in 28 work sections.

2.2. COC collection

2.2.1 Oocyte assays

Culture dishes (GWst-3522, 50x7 mm, WillCo-Wells, Netherlands) were placed in a humidified incubator (THERMO Steri-cycle CO_2 incubator HEPA Class 100) at 38.5°C, 5% CO_2 for a few minutes and subsequently examined using a stereomicroscope equipped with a warm stage (Wild Heerbrugg-M32) at 6-45× magnification. To improve microscopic observation in the presence of debris, the contents of a single dish were divided into additional Petri dishes, diluting the contents

with more medium. Once identified, the COCs were collected using a glass capillary with a mouth pipette and transferred to a 35-mm Petri dish filled with DM.

2.2.2 Morphological classification of COCs

Based on their morphology, the recovered COCs were divided into three categories: 1) oocytes with a compact *cumulus*, 2) oocytes with an expanded *cumulus*, and 3) oocytes with only the *corona radiata*. The three cell categories were kept separate during the following experiments. Some authors have reported maturation rates of 51% and 60% in compact and expanded cumulous, respectively (Galli *et al*. 2007). The morphological criterion used for COC evaluation was based on previous publications (Tharasanit *et al*. 2006, Hinrichs & Williams 1997, Hinrichs 2011). COCs are classified as compact when the *granulosa* appears as sheets of tissue with an even surface that may be thrown into folds by the scraping with a curette. This tissue, as seen on the surface of a fold or on the hillock covering the oocyte, is smooth without evidence of protruding cells. The compact *cumulus*-oocyte complex is typically shaped like a flat-brimmed hat, with a flat underside and a bulging upper surface covered by a "hillock" of *cumulus*.

Only those oocytes with both compact *cumulus* and compact *granulosa* are classified as compact. In expanded COCs, the *granulosa* is not in smooth sheets or flat layers. This can include some cells protruding from a sheet, to sheets with a thickened or puffy granular appearance, to clouds of expanded cells. Very expanded *granulosa* may be associated with a jelly-like yellow substance. Compact *granulosa* originates from viable follicles, whereas expanded *granulosa* originates from atretic follicles. In other species, oocytes with expanded *granulosa* at the time of collection are discarded as degenerated. However, both types of horse oocyte are valuable (Hinrichs & Williams 1997, Hinrichs 2011) (Figure 1).

The oocytes are exposed to mechanical trauma during collection and washing, and partial or total loss of the *cumulus* can occur. This condition was observed in the oocytes with an incomplete *cumulus* or only the *corona radiata*.

The gametes with a diaphanous aspect and prominent nucleus or with evident abnormalities (poor and inhomogeneous cytoplasm, fragmented and pyknotic nucleus) were excluded from the experiment because of non-viability.

2.3. IVM

The COCs considered morphologically suitable for IVM were transferred to different four-well dishes filled with 500 μL maturation medium (MM). Approximately 10 COCs were placed in each well. The category division (compact, expanded, and *corona radiata*) was maintained to evaluate the difference in IVM response between COCs categories. The MM used was M199 (9.70 g/L) modified with the addition of fetal calf serum (FCS, 10%), epidermal growth factor (EGF, 0.0005 g/L), lactate (7.40 g/L), cystine (0.09 g/L), cysteamine (0.04 g/L), myo-inositol (0.005 g/L), ascorbic acid (0.07 g/L), glutamine (0.09 g/L), sodium pyruvate (0.10 g/L), insulin-transferrin-sodium selenite (ITS, 0.001 g/L), and menotropin (Menogon®, Ferring S.p.a, 46.95 UI/L) adjusted to pH 7.2. The complete process was performed within 5 h at a room temperature that ranged between 22 and 26°C to prevent the impairment of vitality and maturation competence (Pedersen *et al*. 2004).

Homogeneous groups of the three categories of COCs, consisting of approximately the same number or proportion of compact, expanded, and corona radiata COCs, were assigned to different incubation times (24, 36 or 45 h) and placed in the incubator at 38.5°C with 5% CO_2. The purpose was to determine if 36-h maturation time under our laboratory conditions was suitable to obtain adequate maturation percentages because only 36-h matured COCs were submitted for PLM observation. The COCs incubated for 24 and 45 h were only submitted to morphological examination by stereomicroscope at 60× magnification (for evaluation of cumulous morphology, cytoplasm segregation of dark and light zones, presence of polar body), and the results were compared with 36-h matured COCs.

2.3.1 Oocyte decumulation

According to previously described criteria, the COCs were evaluated at the end of the assigned maturation period for morphologic assessment of the *cumulus* (expanded or still compact) (Hinrichs & Williams 1997, Hinrichs & Schmidt 2000). After COC observation, the *cumulus* was removed. Decumulation is a necessary step for microscopic evaluation of the polar body and nucleus and for subsequent PLM analysis of oocytes. Enzymatic digestion with a hydrolyzing solution combined with mechanical aspiration into decreasing diameters of glass capillaries was used to fragment the junctional desmosome of the *cumulus*. The procedure was repeated four times in four different 35-mm Petri dishes maintained at a temperature of 38.5°C and filled with 2 mL of four different types of media: (1) HEPES synthetic oviductal fluid (H-SOF) (Tremoleda *et al*. 2003) supplemented with 20 µL hyaluronidase (0.0025 g/L); (2) trypsin (2.50 g/L) buffered with HEPES (4.76 g/L) and supplemented with EDTA (0.40 g/L), d-glucose (1 g/L), NaCl (7 g/L), Na_2HPO_4 (0.12 g/L), KH_2PO_4 (0.24 g/L), and KCl (0.37 g/L); (3) H-SOF supplemented with 200 µL FCS; and (4) H-SOF. Non-essential MEM (10 mL/L) and essential MEM (20 mL/L) buffered with HEPES (4.76 g/L) and supplemented with $CaCl_22H_2O$ (0.25 g/L), $MgCl_26H_2O$ (0.10 g/L), NaCl (6.77 g/L), KCl (0.30 g/L), KH_2PO_4 (0.16 g/L), $NaHCO_3$ (0.42 g/L), sodium pyruvate (0.036 g/L), penicillin (0.063 g/L), streptomycin (0.050 g/L), sodium lactate (0.74 g/L), D-glucose (0.27 g/L), BSA fraction V (4 g/L), glycine (0.75 g/L), and alanyl glutamine (0.22 g/L) were utilized to obtain H-synthetic oviductal fluid (H-SOF) medium. All the reagents were purchased from Sigma-Aldrich (USA).

First, the COCs were transferred by gentle aspiration into a glass capillary to stimulate *cumulus cell* removal by mechanical action from the MM into the hyaluronidase-supplemented medium (medium 1) to ensure standardized exposure to the enzymatic medium and obtain the first digestion of cell junctions. The oocytes were then placed into the trypsin solution (medium 2) for no longer than 90 s to further reduce cohesion between *cumulus* and *corona radiata* cells. To ease the final denudation, COCs were maintained in medium 3 and aspirated into progressively decreasing diameter capillaries to allow the mechanical detachment of the *corona radiata* cells from the *pellucida*. In the

last Petri dish (medium 4) the oocytes were rinsed for few minutes to remove the remaining cellular debris and any traces of enzymatic solutions.

The oocytes to be submitted to PLM analysis were selected at the end of the IVM period on the basis of morphological characteristics. Cells with degeneration marks (e.g., the presence of ZP anomalies and/or the presence of cytoplasmic fragments in the perivitelline space or pyknotic nucleus) were excluded from the study. The selected oocytes (n=127) grouped in respect to the initial *cumulus* morphology were placed in Eppendorf tubes with 300 μL TCM-199 supplemented with gentamicin (0.05 g/L), sodium pyruvate (0.022 g/L), $NaHCO_3$ (0.35 g/L), and 10% FCS at 38.5°C and transported in an insulated flask to the FIVER laboratory of Sant'Anna Hospital, University of Turin, Italy.

2.4. PLM analysis

For the non-invasive evaluation of oocyte quality, PLM analysis was carried out using a polarized light microscope (Polscope) equipped with Oosight™ software to automatically acquire the zona pellucida (ZP) birefringent membrane measurements. The PLM technique allows detailed visualization of the ZP structure and identification of three ZP layers (inner, IL; outer, OL; and middle layer, ML). Our investigation aimed to specifically analyze the IL-ZP, which is characterized by greater birefringence.

Maintaining the initial *cumulus* morphology grouping, the following parameters were considered: (i) total area and thickness of the IL-ZP; (ii) retardance of the IL-ZP; and (iii) total ZP thickness. Retardance is considered an expression of structural density; the highest retardance values correspond to the highest density values. The observations were performed with an inverted microscope (Nikon Eclipse TE 2000-S) equipped with a LC PolScope controller (CRI, USA), a color-coded Doppler (CCD) camera, and a heated ceramic plate. The images were acquired at 40× magnification, and Oosight™ software was used to obtain the measurements. The oocytes were placed in 8-μL drops of equilibrated buffered medium (Gamete Medium, Cook, Ireland) covered

with 3 mL equilibrated paraffin oil (Oil Medium, Cook, Ireland) in glass Petri dishes (GWst-3522, 50×7 mm, WillCo-Wells, Netherlands). To achieve optimal visualization of the anisotropic structures during the examination, oocytes were rotated using an injection pipette (Injection Pipette, Cook, Ireland) to place the polar body and ZP on the same axis. To measure ZP thickness, a line between the innermost (contiguous with the *oolemma*) and outermost point of the ZP was drawn. The measurements were taken twice per each sample to obtain average records, and the images were collected. Due to the heterogeneous characteristics of the ZP, the measurements expressed in micrometers were taken at four different points to ensure accurate assessment, and the mean of these four values was used for statistical analysis (Kilani *et al*. 2006).

2.5. Parthenogenetic activation

According with the literature, to demonstrate the final viability of oocytes matured for 36 h and after PLM evaluation (Fernandes *et al*. 2014), 167 oocytes were submitted to parthenogenetic activation using ionomycin- and 6-dimethylaminopurine (6-DMAP)-supplemented medium as activating agents. First, the oocytes were transferred for 5 min into 3.5-mm Petri dishes with 5 mL TCM-199 with FCS 10% added with 10 μL ionomycin (1.85 g/L). Then, the gametes were washed several times in TCM-199 supplemented with 10% of FCS and incubated in another 3.5-mm Petri dish with 5 mL TCM-199 with FCS 10% and 2 mg 6-DMAP for 5h at 38.5°C under 5% CO_2 in air. At the end of the incubation, the oocytes underwent serial washes in TCM-199 supplemented with 10% FCS. The cells were then placed in 10-µL drops of SOF (Tremoleda *et al*. 2003), covered with equilibrated paraffin oil in 35-mm glass Petri dishes, and incubated in a jar at 38.5°C with 5% O_2 and 5% CO_2 for 2 to 6 days. To assess the activation progress, dishes were examined daily to verify achievement of the first cleavage and cleavage progression timing. Non-essential MEM (10 mL/L) and essential MEM (20 mL/L): $CaCl_2 2H_2O$ (0.25 g/L), $MgCl_2 6H_2O$ (0.10 g/L), NaCl (5.40 g/L), KCl (0.53 g/L), KH_2PO_4 (0.16 g/L), $NaHCO_3$ (2.20 g/L), HEPES (4.76 g/L), Na-pyruvate (0.036 g/L), penicillin (0.063 g/L), streptomycin (0.050 g/L), Na-lactate (0.37 g/L), D-glucose (0.27 g/L),

BSA FAF (bovine serum albumin fatty acid free, 4 g/L), glycine (0.75 g/L), and alanyl glutamine (0.22 g/L) were utilized to obtain SOF medium.

2.6. Statistical analysis

The results are expressed as mean ± standard deviation and are graphically represented in histograms. Statistical analysis was performed using GraphPad InStat (GraphPad Software, USA). Student's t tests were used to compare two sets of quantitative data when samples were collected independently from one another (for unpaired samples), and non-parametric Mann-Whitney U tests were used to compare non-Gaussian variables.

Analysis of variance (ANOVA) for parametric data and Kruskal-Wallis tests for non-parametric data followed by an appropriate post-hoc test (Bonferroni or Dunn's tests, respectively) was used to compare more than two data sets. To choose between parametric or non-parametric tests, sample distributions were evaluated using the Kolmogorov-Smirnov test. In all of the analyses, differences were considered statistically significant when $p<0.05$.

3. Results

3.1. COC collection and IVM

A total of 922 oocytes were collected from 1,331 dissected follicles from 187 processed ovaries during 28 work sessions. In the absence of ovarian structures, ovaries (n=21) were withdrawn from the study. The 805 COCs included in this study were processed during 24 work sessions (85.71%). The average number of dissected follicles per ovary was 7.90±3.05, and the average number of collected oocytes per ovary was 5.42±2.61 with 0.69±0.10 recovered oocytes for each dissected follicle and a recovery percentage of 68.82±10.31% (Table 1). The 922 collected COCs were classified as expanded (461 oocytes, 50%), compact (330 oocytes, 35.79%), *corona radiata* (96 oocytes, 10.41%), and nude or degenerated (35 oocytes, 3.80%) depending on the *cumulus* presence/absence and morphology. Among all COCs, 805 (87.31%) were selected for IVM. The

gametes were classified and grouped based on *cumulus* characteristics: 300 compact COCs (37.27%), 421 expanded COCs (52.30%), and 84 (10.43%) COCs showing only the *corona radiata*.

3.2. IVM

3.2.1 Maturation output of the three groups

The groups submitted to three different maturation times (24, 36, or 45 h) were as follows: 71 COCs submitted to the 24-h maturation protocol (29 compact *cumulus*, 40.84%; 33 expanded *cumulus,* 46.48%; and 9 *corona radiata,* 12.68%), 537 COCs submitted to the 36-h maturation protocol (203 compact *cumulus,* 37.80%; 283 expanded *cumulus,* 52.70%; and 51 *corona radiata,* 9.50%), and 197 COCs submitted to the 45-h maturation protocol (68 compact *cumulus,* 34.52%; 105 expanded *cumulus,* 53.30%; and 24 *corona radiata,* 12.18%). To verify the maturation rate, after decumulation, which took place at the end of each maturation time (24, 36, or 45 h), all the oocytes were observed at 60× magnification using a stereomicroscope equipped with a warm stage (Wild Heerbrugg-M32). In group 1 (24 h), 22 (30.99%) mature oocytes were detected, (*i.e*. oocytes showing both cytoplasmic [cytoplasm segregation of dark and light zones] and nuclear maturity [presence of polar body extrusion as a consequence]), with an average of 5.5±3.32 oocytes per maturation session. In group 2 (36-h incubation), 253 (47.11%) mature oocytes were observed with an average of 16.87±6.23 oocytes per maturation session. In group 3 (45-h incubation), 73 (37.06%) mature oocytes with an average of 14.60±4.34 oocytes per maturation session were detected. The average percentages of maturation per session in groups 1, 2, and 3 were 29.31±13.85%, 47.01±9.90%, and 36.62±5.28%, respectively. Finally, the average percentages of immature oocytes per work session were 28.78±20.17%, 7.83±5.51%, and 22.36±8.39% in group 1 (Table 2), group 2 (Table 3), and group 3 (Table 4), respectively.

3.2.2 Comparison between IVM incubation times

We statistically compared the average maturation percentage and average percentage of oocytes that failed to mature. Data were grouped by single work session and related to the different IVM incubation times. In our laboratory conditions, the IVM protocol with 36-h incubation (group 2) showed the best output in terms of maturation percentage per single session. Specifically, the differences between the percentages obtained from groups 1 and 2 were significant ($p<0.05$). Regarding the average percentages of immature oocytes per single work session, the differences between values obtained from group 2 and the other two groups were significant ($p<0.05$).

3.3. PLM analysis

3.3.1 Mature and immature oocytes analysis

A group of 127 oocytes randomly selected from the 537 oocytes subjected to 36-h IVM was analyzed using polarized light microscopy. Of these oocytes, 36 were immature, and 91 were mature with evident polar body extrusion (Figure 2). Based on ZP birefringence parameters, the oocytes were subdivided into mature or immature, and the results were compared (Figure 3, 4, 5, and 6). The average retardance showed a significant increase ($p<0.01$) in immature oocytes (2.71±1.06 nm) compared with that for mature oocytes (2.12±0.54 nm). Similarly, the average ZP thickness was significantly increased ($p<0.001$) in immature oocytes (20.07±2.93 μm) compared with that of mature oocytes (17.90±2.53 μm). No statistically significant differences were recorded between the two groups in terms of the area or thickness of the IL-ZP.

3.4. Activation

Quality and meiotic competence of the oocytes after IVM were evaluated based on parthenogenetic activation of the oocytes subjected to 36 h of maturation, which were previously analyzed using the Polscope (Caamaño *et al*. 2010, Carneiro *et al*. 2001).

A total of 167 metaphase II oocytes (91 PLM observed and 76 randomly selected), derived from 70 compact COCs (41.92%), 79 expanded COCs (47.30%), and 18 *corona radiata* cells (10.78%) were

parthenogenetically activated. Eighty-nine oocytes showed signs of activation, with an average of 57.28±22.62% (range, 17.39% to 85.71%) activated cells per session. The average activation percentages among different cell populations were evaluated every 24 h per single session as described previously and confirmed as positive when two-cell division was observed. Expanded COCs showed a greater activation percentage (61.83±13.34%) compared with *corona radiata* (27.08±29.46%) and compact COCs (57.53±34.85%). Comparing the expanded and *corona radiata* oocytes, the difference between average activation percentages per single session was significantly ($p<0.05$) in favor of expanded oocytes, which revealed good reproducibility per session.

3.4.1 Differences in successfully and unsuccessfully activated oocytes observed by PLM.

Immediately after PLM analysis, 91 oocytes subjected to IVM for 36 h and observed by PLM underwent parthenogenetic activation. The percentage of oocytes that showed embryonic cleavage (activation rate) was 67.03% (n=61). The values obtained from PLM examination (Figure 7) of activated and non-activated oocytes were compared and statistically analyzed, and the results were represented graphically (Figure 3, 4, 5, and 6). The difference between the average IL-ZP area of activated oocytes (2,394.23±469.82 μm^2) and that of non-activated oocytes (2,075.81±291.62 μm^2) was significant ($p<0.001$). The average IL-ZP thickness of activated and non-activated cells was 4.68±1.07 and 3.89±0.72 μm, respectively, ($p<0.01$). Conversely, the IL-ZP retardance and ZP thickness were not significantly different between the two groups of oocytes. The average retardance measurement among activated oocytes was 2.18±0.55 nm, and the average ZP thickness was 18.14±2.37 μm, whereas the average retardance and ZP thickness measured among non-activated oocytes were 2.00±0.48 nm and 17.40±2.79 μm, respectively.

4. Discussion

Polarized light microscopy offers the unique advantage of being a completely noninvasive assessment; it is able to preserve oocyte viability and allows repeated observations of a sample. The PLM has the capability to emphasize and measure the molecular order of birefringent objects. The

molecular order of the body is quantified by measuring the change in phase between the two polarized waves emerging from the object, and this parameter is called retardance. This and other ZP parameters linked to its structure are correlated with gamete fertility and with the ability of the oocyte to have better development after IVF. The majority of studies have been performed on human, mice, and bovine oocytes (Caamaño *et al*. 2010, Koester *et al*. 2011, Rama Raju *et al*. 2007). So far, only one study regarding the Polscopic evaluation of equine oocytes has been performed (Mohammadi-Sangcheshmeh *et al*. 2014). The literature presents conflicting data regarding the correct incubation time required to induce complete IVM of equine oocytes. In our working conditions, the 36-h incubation period was identified as the adequate culture time to obtain the highest number of oocytes characterized by full cytoplasmic and nuclear maturity (morphologically evaluated) and the lowest number of immature oocytes, compared with the 24- and 45-h incubation times. The percentages of cells matured *in vitro* using the 36-h protocol were higher than the percentages achieved employing the 24-h incubation time (47.11% vs. 30.99%, $p<0.05$). These results were similar to the values obtained by other groups (Willis *et al*. 1991, Dell'Aquila *et al*. 1997a, Hinrichs & Schmidt 2000, Galli & Lazzari 2001, Galli *et al*. 2002, Lagutina *et al*. 2005, Galli *et al*. 2007). Oocytes matured for 36 h, presenting full cytoplasmic and nuclear maturation (with polar body extrusion), were subjected to parthenogenetic activation immediately after PLM analysis, which confirmed that oocyte quality and meiotic competence were adequate after 36 h of IVM. These results are also correlated with the birefringence analysis of the ZP.

We analyzed the correlation between oocyte developmental potential (in terms of percentage of oocytes that achieved *in vitro* activation) and the initial light microscopy morphological classification of 36-h matured oocytes. In our study, an average percentage of cleaved oocytes per session of 57.28±22.62% was obtained. This value was significantly higher than percentages reported in published literature for equine oocytes (28.5%) (Carneiro *et al*. 2001). Among the analyzed oocytes, the average activation percentage per session of the expanded oocyte group

(61.83±13.34%) was higher than those of the compact group (57.53±34.85%) and the *corona radiata* group (27.08±29.46%). The differences between the activation percentages of the *corona radiata* and expanded oocytes were significant ($p<0.05$). These data are in accordance with the results obtained by Mohammadi-Sangcheshmeh *et al*. in 2014 after intracytoplasmic sperm injection (ICSI). They obtained 50.7% cleaved oocytes among expanded COCs, even though this result was not statistically significant. The oocytes derived from expanded COCs, matured 36 h, and activated showed the best ability to be stimulated by parthenogenetic activation. Several works have reported that developmental competence differs between expanded and compact equine oocytes (Hinrichs *et al*. 1995, Choi *et al*. 2004, Dell'Aquila *et al*. 1997b, Hinrichs *et al*. 2002, Franz *et al*. 2003, Hinrichs & Williams 1997). Nevertheless, these differences have not been completely characterized or understood because of the complexity of the events, and the large number of involved factors, such as maturation conditions and embryo culture media composition (Choi *et al*. 2004). Therefore, it cannot be unequivocally stated that expanded oocytes are best suited for fertilization/activation procedures.

We also analyzed the correlation between oocyte developmental potential, zona pellucida birefringence, IL-ZP area and thickness, and total ZP thickness. This allowed us to compare the subjective morphological evaluation obtained using classic light microscopy on 36-h mature and immature oocytes with objective computerized evaluations obtained with PLM. The retardance of the IL-ZP and total thickness of the ZP were significantly increased in immature oocytes compared with that of mature oocytes. The average retardance values for immature and mature oocytes were 2.71±1.06 and 2.12±0.54 nm, respectively ($p<0.01$). The average value of the total ZP thickness was 20.07±2.93 μm in immature oocytes and 17.90±2.53 μm in mature oocytes ($p<0.001$). These findings are in accordance with previously reported bovine oocyte data (Koester *et al*. 2011; Held *et al*. 2012). These studies revealed that more advanced maturation stages corresponded to lower birefringence values of the ZP, contrary to what was observed in human oocytes (Shen *et al*. 2005, Rama Raju *et al*. 2007). Our results are in disagreement with other studies in which expanded

oocytes with high developmental capacity, determined by cumulus morphology, had a significantly thicker and higher ZP retardance compared with compact oocytes (Mohammadi-Sangcheshmeh *et al*. 2014). Moreover, that group found that oocytes characterized by higher developmental competence (assessed by evaluation of the glucose-6-phosphate dehydrogenase activity with brilliant cresyl blue testing) had a significantly thicker ZP and higher ZP birefringence.

Parthenogenetic activation also allowed us to assess whether the parameters measured after IVM using PLM were different in activated oocytes compared to non-activated oocytes and thus to correlate the activation ability with these parameters. This evaluation revealed that both the area and thickness of the IL-ZP are significantly increased in oocytes that responded positively to the activation procedure. The average IL area of activated oocytes was 2,394.23±469.82 μm^2, while it was 2,075.81±291.62 μm^2 for non-activated oocytes, ($p<0.001$). The average value of the IL-ZP thickness was 4.68±1.07 μm in activated oocytes and 3.89±0.72 μm in non-activated oocytes ($p<0.01$). These values could thus be considered predictive of achieving an adequate maturation status and good meiotic competence for oocytes after IVM. However, no statistically significant differences were found in IL-ZP retardance or total ZP thickness between the two groups, although we noticed a tendency toward increased values in activated oocytes.

In human medicine, there are many conflicting reports regarding thickness and area of the inner layer of the ZP. In a previous work, the IL-ZP area of both oocytes and embryos was significantly lower in conception than in non-conception cycles, whereas IL-ZP retardance values of oocytes and embryos were comparable in conception and non-conception cycles (Molinari *et al*. 2012). In contrast, Shen and colleagues (2005) demonstrated that IL-ZP thickness was slightly elevated and mean IL-ZP retardance was higher in oocytes contributing to conception cycles compared to non-conception cycles. Nevertheless the ZP and spindle morphology measured by polarized light microscopy can be considered a prognostic marker for oocyte quality (Wang *et al*. 2001a, Wang *et al*. 2001b, Cooke *et al*. 2003, Keefe *et al*. 2003, Moon *et al*. 2003, Cohen *et al*. 2004, Pellettier *et al*. 2004, Rienzi *et al*. 2004, Trimarchi *et al*. 2004, Rama Raju *et al*. 2007, Ebner *et al*. 2008, Montag &

van der Ven 2008, Madaschi *et al*. 2009). Specifically, the inner layer of the ZP is crucial for embryonic development, and higher IL-ZP thickness and retardance values are related to greater potential for oocyte development (Shen *et al*. 2005, Rama Raju *et al*. 2007). In our study, IL-ZP area and thickness were increased in positive activated oocytes in accordance with reports by Shen *et al*. (2005) and Rama Raju *et al*. (2007).

The equine oocyte is characterized by the presence of abundant cytoplasmic granulation (Caamaño *et al*. 2013), which often prevents meiotic spindle display. Therefore, unlike in human and bovine oocyte analyses, the investigation of equine gametes could be based solely on evaluation of the ZP.

Successfully activated bovine oocytes showed lower retardance values compared with oocytes that failed cleavage (Koester *at al*., 2011). Similarly, fertilized oocytes that cleaved exhibited lower birefringence/retardance values compared to their counterparts that failed fertilization. Those data are in contrast with our findings, even if our results were not statistically significant.

Polscopic analysis of birefringent oocyte structures revealed predictive values for the quality and competence of equine, bovine, and human oocytes, which are related to the results of parthenogenetic activation. These findings suggest that greater use of polarized light microscopy in veterinary medicine could improve the selection of good quality oocytes subjected to further *in vitro* fertilization techniques. In particular, the use of PLM in assisted reproduction in equines could be helpful to obtain greater success rates in ICSI cycles; those recorded to date have an average percentage of blastocyst formation of 5-15% (Choi *et al*. 2004). By only selecting gametes with the best morphological characteristics, which are related to ZP birefringence, to be submitted for further fertilization techniques, better fertilization results could be expected.

In conclusion, our paper proves that: a) after IVM, the equine oocytes can be evaluated using classical microscopy for the observation of morphological parameters as well as using PLM; b) the PLM is able to detect differences between mature and immature oocytes, and between mature oocytes that undergo activation and mature oocytes that do not activate; c) IVM of equine oocytes

causes changes in the inner layer structure of the ZP; d) the parthenogenic activation generate additional changes in this structure.

Then, on a purely speculative level, it can be assumed that these variations, detectable by PLM, could be used to predict the ability of equine oocytes to be activated and that the predictive capacity of PLM could be higher than that of morphological evaluation obtained using a classic light microscope, but more studies are required to prove it.

5. Declaration of interest

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

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9. Figure legends

Figure 1 Compact (A), expanded (B), and corona radiata COCs (C). BAR= 100 μm.

Figure 2 Immature oocyte (A) and oocyte showing signs of cytoplasmic and nuclear maturation (B). BAR= 100 μm.

Figure 3 Graphical representation of the IL-ZP area values in immature/mature and non-activated/activated oocytes.

Figure 4 Graphical representation of the IL-ZP thickness in immature/mature and non-activated/activated oocytes.

Figure 5 Graphical representation of the IL-ZP retardance in immature/mature and non-activated/activated oocytes.

Figure 6 Graphical representation of the ZP thickness in immature/mature and non-activated/activated oocytes.

Figure 7 PLM analysis of the zona pellucida: measurement of the area and retardance of the ZP (A) and measurement of the ZP thickness (B). BAR= 100 μm.

10. Tables

11. Figures