Glutathione Content and Glutathione Peroxidase Expression in In Vivo and In Vitro Matured Equine Oocytes

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ABSTRACT
The in vitro developmental competence of equine oocytes is still low in comparison with other domestic mammals. A major factor affecting the viability of cells during in vitro culture is the increased oxidative stress. Oxidative modifications could be responsible for oocyte incompetence for in vitro maturation (IVM). Cysteamine, a glutathione (GSH) synthesis enhancer, has been shown to increase intracellular GSH content and to improve embryo development when added during IVM of bovine, porcine, and ovine oocytes. The aim of the present study was (1) to determine whether equine cumulus–oocyte complexes (COCs) benefit from the addition of cysteamine during IVM, (2) to compare the GSH content of oocytes after in vivo maturation and IVM, (3) to assess whether cysteamine administration during IVM of equine oocyte enhances early embryonic developmental capability following ICSI, (4) to study the glutathione peroxidase (GPX) mRNA level in COCs. In vivo matured COCs were collected by aspiration from preovulatory follicles, and analyzed at collection. Immature COCs were collected in vivo or from slaughterhouse ovaries and matured in culture media supplemented or not with 100 μM cysteamine. After nuclear stage assessment, oocytes were analyzed for GSH concentration and both oocytes and cumulus cells were analyzed for GPX and GAPDH mRNA. Our data showed that the maturation capability was similar in both in vivo aspirated oocytes and in those isolated from slaughterhouse ovaries. Moreover, the addition of cysteamine during IVM affected neither GSH content nor maturation rate. At the time of collection, intracellular GSH content was not influenced by the chromatin status. GSH concentration was similar in both in vivo and in vitro matured metaphase II (MII) stage oocytes, and was significantly higher in MII than immature germinal vesicle stage oocytes. Moreover, the presence of serum inhibited whereas its absence stimulated the accumulation of GSH within oocytes during IVM. After ICSI, a similar proportion of zygotes in each group developed beyond the two-cell stage after 72 hr of culture. Cumulus cells expressed GPX mRNA, while GPX transcript was absent in both immature and mature oocytes. Cumulus expression of GPX mRNA was significantly higher when analyzed at collection than after IVM. Taken together, our results demonstrate that in equine oocytes, GSH increases during IVM but the relative intra-oocyte content of this thiol does not affect maturation and early development efficiency after fertilization. We hypothesize that factor(s) other than GSH/GPX are responsible for the limited in vitro early developmental capability of equine oocytes. Mol. Reprod. Dev. 73: 658–666, 2006. © 2006 Wiley-Liss, Inc.

Key Words: horse; oocyte; cumulus cells; germinal vesicle; chromatin; glutathione; glutathione peroxidase

INTRODUCTION
In the recent years, assisted reproduction technologies (ART) outputs have improved the reproductive performances in mammals. In bovine species, extensive research has been carried out in the field of in vitro embryo production (IVP), and satisfactory results are now routinely achieved for in vitro oocyte maturation (IVM), spermatozoa capacitation, and in vitro fertilization (IVF) techniques. On the contrary, ART have produced only limited successes in equine species. A possible reason could be the lack of detailed information about the physiological mechanisms that control follicle development, oocyte maturation, and fertilization in this species.

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Previous studies have shown that the low IVP success rate in the equine species is mainly due to the large proportion of oocytes that fail to reach a complete cytoplasmic and nuclear maturation during in vitro culture (Goudet et al., 1997; Hinrichs, 1998; Squires et al., 1999). Recent studies have pointed out that several factors could contribute to this heterogeneity such as seasonality (Hinrichs and Schmidt, 2000; Colleoni et al., 2004) as well as intrinsic factors such as the size of the follicle, cumulus oophorus morphology, germinal vesicle chromatin configuration or the degree of follicular cells apoptosis (Goudet et al., 1997; Hinrichs and Williams, 1997; Hinrichs and Schmidt, 2000; Dell’Aquila et al., 2003).

Recent studies indicate that in mouse and human, oxidative stress could be an important factor affecting the oocyte viability during in vitro culture (Guerin et al., 2001). Glutathione (GSH) is the major nonprotein sulfhydryl compound in mammalian cells and is well known to play an important role in protecting cells against the destructive effects of reactive oxygen intermediates and free radicals, collectively named reactive oxygen species (ROS) (Meister, 1983). GSH also acts as a cofactor for enzyme activity, regulates protein and DNA synthesis by altering redox status, and participates in microtubule assembly (Denneke and Fanburg, 1989). In reproductive function, GSH participates in sperm capacitation and male pronucleus formation, during fertilization in hamster (Perreault et al., 1988), pig (Yoshida et al., 1993), and cow (Sutovsky and Schatten, 1997). The addition of GSH synthesis precursors such as cysteine or cysteamine to the culture medium improves the maturation efficiency by increasing the oocyte GSH content in mouse (de Matos et al., 2003), pig (Abeydeera et al., 1999), cow (de Matos et al., 1995), sheep (de Matos et al., 2002a), and buffalo (Gasparini et al., 2003). Cumulus cells play an important role in GSH synthesis because the increase in oocyte GSH level is dependent on the presence of cumulus mass while denuded oocytes have only a limited capability to synthesize GSH (de Matos et al., 1997).

Various metabolic pathways and enzymes can produce ROS, including mainly oxidative phosphorylation (Thompson et al., 2000). However, free oxygen radicals can be generated by the time of oocyte isolation from the follicle environment in the practice of ART (Iwata et al., 2003). Several complementary mechanisms are involved in protecting gametes from ROS. In vivo, oocytes and embryos seem to be protected against oxidative stress by oxygen scavengers present in follicular and oviduct fluids (Carbone et al., 2003; Lapointe and Bilodeau, 2003). Glutathione (GSH) appears to be the main nonenzymatic defense system against ROS (Gardiner and Reed, 1994), and is also the substrate of glutathione peroxidase (GPX), the main antioxidant enzyme, playing a pivotal role in cell antioxidant protection from lipid hydroperoxides and H$_2$O$_2$ (Guerin et al., 2001). GPX transcripts are detected in mouse and human MII oocytes but not in germinal vesicle stage human gametes (El Mouatassim et al., 1999), while in bovine, both in vivo and in vitro matured oocytes displayed a similar relative abundance, significantly higher than immature oocyte (Lonergan et al., 2003a). To date, no information is available in horse.

Certainly, in vitro maturation (IVM) conditions of equine oocytes need to be refined and a better understanding of the factors, which modulate the acquisition of a complete nuclear and cytoplasmic maturation, may be helpful in the development of strategies to improve culture conditions and reproductive technologies in this species. The present study was designed (1) to determine whether equine cumulus–oocyte complexes (COCs) benefit from the addition of cysteamine during IVM in different culture conditions, (2) to compare the GSH content of oocytes after in vivo maturation and IVM, (3) to assess whether cysteamine administration during IVM of equine oocytes enhances early embryonic developmental capability following ICSI, (4) to evaluate the glutathione peroxidase expression in equine COCs.

**MATERIALS AND METHODS**

**Chemicals and Reagents**
All chemicals and reagents were purchased from Sigma Chemical Company (St. Louis, MO), unless otherwise stated.

**Cumulus–Oocyte Complexes Isolation**
Equine COCs were collected either by transvaginal ultrasound-guided aspiration in standing mares or from ovaries recovered from slaughtered mares during the breeding season.

Adult cycling pony mares in good body condition, kept indoor and fed with concentrates, were used for transvaginal ultrasound-guided punctures. Ovarian activity was assessed daily by 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 (Duchamp et al., 1987). All follicles larger than 5 mm were punctured 24–34 hr after induction of ovulation, as previously described (Goudet et al., 1997). During follicle puncture, mares were sedated with detomidine (Domosedan, 0.15 ml/animal, i.v., Pfizer, Amboise, France) and the rectum was relaxed with propantheline bromide (60 mg/animal 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 fluids (follicular fluid and rinsing) from each follicle were examined individually with a stereomicroscope for COC recovery. After puncture sessions, mares received an antibiotic injection (Intramicine, 4 g benzylpenicillin procaine/animal, and 4,000,000 IU dihydrostreptomycin/animal i.m., Ceva, France).

Ovaries from mares of unknown reproductive history were recovered from local abattoir, and immediately transported after slaughter to the laboratory within 1 hr in saline at 32–34°C. The tunica albuginea was removed and all follicles larger than 5 mm were aspirated with a
16-gauge needle mounted on an aspiration pump (COOK-IVF, Brisbane QLD, Australia) with a vacuum pressure of ~28 mmHg. The ovaries were cut into thick sections with a scalpel blade in order to aspirate follicles eventually present within the ovarian stroma. Follicular fluids were diluted approximately 1:10 with saline supplemented with heparin (501 IU/ml) and COCs recovered under a stereomicroscope.

**Equine Cumulus–Oocyte Complexes Maturation and Nuclear Examination**

Only COCs classified morphologically at recovery as compact or early expanded were used (Dell’Aquila et al., 2004). COCs were washed four times in PBS with gentamycin (50 mg/L, Sigma) and once in the maturation medium. The basic maturation medium (bMM) was TCM 199 with Earle’s salts supplemented with 0.68 mM L-glutamine, 25 mM NaHCO3, 0.2 mM of sodium pyruvate. The basic maturation medium was supplemented with 20% fetal calf serum (FCS) and 50 ng/ml of epidermal growth factor (EGF) (complete medium, cMM) or 0.4% of BSA and 50 ng/ml of EGF (defined medium, dMM).

In the present study, a concentration of 100 μM of cysteamine was used because it has been demonstrated the effective concentration in several mammals (Kito and Bavister, 1997; Gasparrini et al., 2000; Bogliolo et al., 2001; Bogliolo et al., 2002; Rodriguez-Gonzalez et al., 2003; Accardo et al., 2004; Whitaker and Knight, 2004; Kobayashi et al., 2005).

Oocytes were matured in either 500 μl of cMM or cMM supplemented with 100 μM cysteamine, or in dMM with or without 100 μM cysteamine and supplemented either with equine FSH (eFSH, 1 μg/ml, NIH, A.F. Parlow, AFP5022B n’845) or not. COCs were cultured for 30 hr at 38.5°C and 5% CO2 in humidified air.

After culture, COCs were rinsed in PBS and stripped of their cumulus cells with a small glass pipette as previously described (Dell’Aquila et al., 2004). Totally denuded oocytes were rinsed in PBS, stained with 1 μg/ml bisbenzimidle fluorescent dye (Hoechst 33342) in PBS, and examined in a drop on a slide under an inverted epifluorescence microscope (Nikon E-600 Nikon Corp., Tokyo, Japan) in order to determine the nuclear stage. Oocytes were considered mature when they had reached metaphase II (MII), showing a polar body and two distinct spots of chromosomes. Oocytes showing either multipolar meiotic spindle or irregular chromatin clumps or no chromatin were considered to be abnormal. Oocytes with fragmented or shrunken cytoplasm were classified as degenerated.

In order to obtain a mature control for GSH determination and GPX analysis, expanded COCs collected from preovulatory follicles (>35 mm, 35 hr after induction of ovulation) were examined for nuclear stage assessment at collection as above described. Only oocytes that reached metaphase II after in vivo maturation were stored for further analysis. As control for immature oocytes, COCs isolated from small follicles (<30 mm) were examined at collection as above indicated. Only oocytes that were at the GV stage were stored for subsequent analysis.

**Determination of Intra-Oocyte GSH Content**

After nuclear chromatin analysis, single denuded oocytes at GV or MII stage were transferred under a stereomicroscope on the bottom of an eppendorf tube with a narrow-bore pipette and the buffer was successively removed by aspiration. Samples were snap frozen in liquid nitrogen and stored at –80°C until assayed. The oocyte GSH content was determined by 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB)-GSH reductase recycling micro-GSH assay according to Baker et al. (1990) and adapted by P. J. Hansen, University of Florida, (online protocol: http://www.animal.ufl.edu/hansen/), with slight modifications (Luciano et al., 2005). Briefly, oocytes were thawed on melting ice and 50 μl of water was added to each 10 sample. Each microtube was then frozen in liquid nitrogen and successively thawed. Each sample was mixed with vortex and centrifuged for 20 sec at 12,000 rpm. This procedure was repeated three times. Standards containing from 0 to 200 pmol of GSH in 50 μl were prepared in water, simultaneously with the samples. Samples and standards were kept on ice until being loaded in the microtitrere plate. A volume of 50 μl of each sample and standard was added in a 96-well microtiter plate. Reaction mixture was freshly prepared with 0.15 mM of DTNB, 0.2 mM of NADPH, and 1.0 IU of GSH reductase/ml (final concentrations) in 0.1M phosphate buffer supplemented with 1 mM of EDTA, pH 7.8. Immediately, 0.1 ml was pipetted in each well and the plate was analyzed at 405 nm in a microtiter plate reader (SpectraCount, Packard, Meriden, CT, USA) with one initial mixing and repeated-reads functions at 2 min intervals for 30 min. The assay was validated by adding increasing numbers of oocytes (5, 10, 20) to a constant volume of extraction medium and recovering a proportional amount of GSH.

**Extraction of Total RNA and RT-PCR for GPX Analysis**

The Tripure Isolation Reagent Kit (Boehringer, Mannheim, Germany) was used to extract total RNA from oocytes and cumulus cells. This one step extraction procedure was derived from the method of Chomczynski and Sacchi (1987) and was performed according to the manufacturer’s recommendations. Briefly, either a single oocyte (immature or in metaphase II at collection, or metaphase II after culture) or cumulus cells pellets were mixed with 100 μl of tripure reagent and added to 60 μl chloroform. After centrifugation at 14,000g for 15 min, RNA in the upper aqueous phase was recovered and precipitated with ethanol and dried. RNA pellets from oocyte and cumulus cells were dissolved in 5 μl and 10 μl of H2O, respectively, and were reverse transcribed (RT) in a final volume of 10 μl and 20 μl, respectively, containing 2 μM of oligo(dT)12–18.
(Amersham Pharmacia, Orsay, France). 1.25 mM of each dNTP, 2.5 mM of MgCl₂, 20 IU of recombinant ribonuclease inhibitor (RNasin), and 100 IU of Moloney murine leukemia virus reverse transcriptase (Promega, Charbonnière, France) were used for the reverse transcriptase reaction performed for 60 min at 37°C. The reaction was performed for products from cumulus cell mRNA, 0.5 μl of reverse transcriptase and 2.5 μM of each dNTP, and 1 IU of recombinant Taq DNA polymerase, in 25 μl of the appropriate Taq buffer (Promega). PCR reactions were performed in a thermal cycler (geneamp PCR system 9700, Perkin Elmer, Boston, MA, USA) and were initiated by 3 min of denaturation at 94°C, followed by 35 cycles each consisting of 30 sec at 94°C, 30 sec at 62°C, and 45 sec at 72°C. PCR specificity was checked by using a positive and a negative control. PCR products were electrophoresed through a 2% agarose gel, stained with 0.2 μg/ml ethidium bromide, and visualized by ultraviolet illumination. The image of each gel was digitalized and the intensity of each band was assessed by densitometry scanning using an image analysis program (ImageJ v1.34). The relative amount of the mRNA of interest was calculated by dividing the intensity of the band for each oocyte or each cell pellet of the different groups by the intensity of the GAPDH band for the corresponding stage. Experiments were repeated at least five times for each mRNA with single oocyte or a cell pellet as starting material.

<table>
<thead>
<tr>
<th>Maturation conditions</th>
<th>Cysteamine</th>
<th>n</th>
<th>% MII</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vivo collected COCs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cMMa</td>
<td>–</td>
<td>51</td>
<td>76.5</td>
</tr>
<tr>
<td>cMM</td>
<td>+</td>
<td>43</td>
<td>79.1</td>
</tr>
<tr>
<td>Slaughterhouse COCs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cMM</td>
<td>–</td>
<td>34</td>
<td>73.5</td>
</tr>
<tr>
<td>cMM</td>
<td>+</td>
<td>40</td>
<td>70.1</td>
</tr>
<tr>
<td>dMMb</td>
<td>–</td>
<td>48</td>
<td>70.8</td>
</tr>
<tr>
<td>dMM</td>
<td>+</td>
<td>46</td>
<td>69.6</td>
</tr>
<tr>
<td>dMM + FSH</td>
<td>–</td>
<td>53</td>
<td>69.8</td>
</tr>
<tr>
<td>dMM + FSH</td>
<td>+</td>
<td>54</td>
<td>68.5</td>
</tr>
</tbody>
</table>

aCMM is composed of bMM, EGF, and FCS.
bDMM is composed of bMM, EGF, and FCS.

Oocyte Evaluation, Semen Preparation, and Intra-Cytoplasmic Sperm Injection (ICSI)

Oocyte and embryo manipulation medium was modified synthetic oviduct fluid (SOF, Tervit et al., 1972) supplemented with 0.3% (w/v) BSA fraction V, fatty acid free, MEM essential and nonessential aminoacids, 0.72 mM of sodium pyruvate, and buffered with 10 mM of HEPES and 5 mM of NaHCO₃ (SOF-R). After 26–28 hr of IVM culture, oocytes were stripped free of cumulus cells by aspirating them through a finely drawn glass pipettes and washed in HEPES buffered SOF-R medium containing 80 IU hyaluronidase/ml. Oocyte morphology was assessed and only oocytes showing an extruded first polar body in the perivitelline space and an intact oolemma were selected and submitted to microinjection. Samples of frozen semen (0.4 ml/straw) from a single ejaculate from a stallion of proven fertility were thawed for 1 min in a water bath at 37°C. Total motility after thawing was 70%, with 50%–60% progressive motility.

Sperm cells for ICSI were prepared by the swim-up procedure in Ca²⁺ free TALP (Parrish et al., 1988) supplemented with 0.4% BSA. Briefly, a straw of frozen semen was washed in 6 ml of Ca²⁺ free TALP and pelleted by centrifugation at 1,500 rpm for 10 min. On the resuspended pellet was stratified 1 ml of Ca²⁺ free TALP supplemented with 0.4% BSA for the swim-up procedure. After 30 min, motile spermatozoa were recovered from the top and diluted at a concentration of 1 × 10⁶ spz/ml in fertilization SOF (Tremoleda et al., 2003) supplemented with 0.6% (w/v) BSA fatty acid free and 10 μg/ml heparin.

Just before ICSI, 1 μl of sperm suspension was added at the top of a line of a 12% solution of polyvinylpyrrolidone (PVP) in fertilization SOF medium placed by the side of the ICSI drop. Only motile spermatozoa, which were able to swim along the border of the PVP solution, were used for sperm injection.

Intracytoplasmic sperm injection was carried out under an inverted fluorescence microscope (Nikon Diaphot Nikon Corp., Tokyo, Japan), equipped with a Narishige microinjection apparatus (Narishige Co Ltd, Tokyo, Japan) that was used to guide the holding and injecting micropipettes (COOK-IVF) into a 25 μl drop of SOF-R covered with mineral oil. Embryo culture medium was SOF buffered with 25 mM of NaHCO₃, supplemented with MEM essential and nonessential aminoacids, 0.72 mM of sodium pyruvate, 2.74 mM of myo-inositol, 0.34 mM of sodium citrate, and with 5% of calf serum (SOF-C). Injected oocytes were rinsed two times in SOF-R then transferred to fresh SOF-C medium covered by mineral oil and incubated at 38.5°C for 72 hr under an humidified gas mixture composed by 5% CO₂, 5% O₂, and 90% N₂. At the end of the culture period, the percentage of embryos, which developed over the two-cell stage, was assessed by counting cell nuclei under fluorescence microscopy after staining them with 0.5 mg/ml of propidium iodide (Luciano et al., 1999).
Statistical Analysis

All experiments were replicated at least five times. Results are expressed as mean ± SEM. Oocyte maturation and embryonic developmental rates were compared with chi-square test. The nonparametric Kruskal–Wallis test was performed using StatXact 4 software (CYTEL, Cambridge, MA, USA) in order to compare the means of intra-oocyte GSH concentration among groups and the means of gene expression signals.

RESULTS

Oocytes GSH Content, Maturation and Developmental Competencies

As shown in Table 1, the nuclear maturation rate after IVM of in vivo collected immature oocytes was not significantly different to that of oocytes isolated from slaughtered ovaries. Moreover, whatever medium used, all treatments showed a similar efficiency of maturation, either in complete or defined conditions. Only oocyte matured with bMM, in absence of stimulation, showed a maturation rate lower than other treatments (51.3, \( P < 0.05 \)). The addition of cysteamine did not influence the percentage of oocytes that reached the MII stage whatever the maturation condition used, either in oocyte isolated by transvaginal ultrasound-guided aspiration or those isolated from slaughtered ovaries.

Some GV stage oocytes were stained with Hoechst 33342 at collection and classified as previously described (Hinrichs et al., 1993; Hinrichs, 1997; Hinrichs and Williams, 1997) in those in which the nucleus fluoresces diffusely (FN), and those in which the chromatin is condensed as a mass within the germinal vesicle (condensed chromatin; CC). No differences were observed in the frequency of FN and CC oocytes between COCs isolated from slaughtered ovaries and in vivo punctured (not shown). The intra-oocyte GSH content in immature oocytes was not influenced by the chromatin status (1.07 ± 0.7 and 1.01 ± 0.6 pmol/oocyte in FN and CC, respectively).

Glutathione content significantly increased during IVM in oocytes cultured in all maturation conditions used in comparison to the GSH content retained in immature GV stage oocytes (Table 2). After IVM in presence of serum, oocytes collected by in vivo punctures or from slaughtered ovaries showed a similar GSH content to oocytes matured in vivo. The addition of cysteamine to the culture medium did not influence the GSH concentration at the end of maturation period when used in presence of serum.

The presence of BSA as protein source in IVM medium stimulates a significantly higher GSH accumulation in oocytes, in comparison to oocytes matured in presence of serum. Moreover, the addition of cysteamine stimulated the augmentation of GSH within oocytes that reached the MII stage when matured in presence of dMM. Finally, the presence of FSH did not influence the intra-oocyte GSH content at the end of maturation (Table 2).

The administration of cysteamine during IVM of equine oocytes has no effect on the early embryonic developmental capability following ICSI. The percentage of zygotes, which developed beyond the two-cell stage, was similar between oocytes matured with or without the GSH synthesis precursor (Table 3).

Expression of Glutathione Peroxidase in Equine Oocytes and Cumulus Cells

Oocytes (GV and MII) as well as cumulus cells (from compact and expanded COCs) were analyzed by RT-PCR for the presence of GPX mRNA. Figure 1 shows the result obtained. An expected band of 184-base pair (bp) signal was observed in cumulus cells. Semi-quantitative

| TABLE 2. GSH Content (Mean ± SEM) in Immature (GV Stage) and MII Oocytes |
|--------------------------|--------------------------|--------------------------|
| Treatments               | Cysteamine               | n            | GSH Content (pmol/oocyte) |
| In vivo collected COCs   | –                        | 10           | 1.25 ± 0.37a              |
| GV at collection         | –                        | 11           | 5.89 ± 0.46b              |
| MII at collection        | –                        | 13           | 4.74 ± 0.46b              |
| After IVM                | +                        | 15           | 5.06 ± 0.43b              |
| Slaughterhouse COCs      | –                        | 11           | 1.16 ± 0.29a              |
| GV at collection         | +                        | 13           | 4.84 ± 0.33b              |
| dMMa                     | +                        | 14           | 5.09 ± 0.37b              |
| dMMb                     | –                        | 15           | 7.87 ± 0.70c              |
| dMM                       | +                        | 18           | 9.89 ± 0.60c,d            |
| dMM + FSH                | –                        | 16           | 8.51 ± 0.63c              |
| dMM + FSH                | +                        | 18           | 10.58 ± 0.64d             |

a,b,c,d are significantly different (\( P < 0.05 \)).
analysis of the signals showed that the GPX signal varied in relation to the type of maturation. Cumulus cells derived from in vitro matured COCs displayed a lower PCR signal than cumulus cells derived from in vivo matured COCs ($P < 0.05$). By contrast, no signal was observed for GPX in oocytes, regardless of nuclear stage and maturation conditions, despite the correct amplification of a 255-bp fragment for GAPDH mRNA in the samples studied, and despite the correct PCR positive control for the target gene.

**DISCUSSION**

This study is the first in the horse to show the role of glutathione and glutathione peroxidase in cumulus–oocyte complex. Analogously to other species (Perreault et al., 1988; de Matos et al., 1997; Gasparrini et al., 2003; Zuelke et al., 2003), also in equine intra-oocyte GSH concentration increases during both in vivo and IVM. Moreover, our study demonstrates that culture conditions stimulate a significant increase in oocyte GSH content during meiotic progression, from GV stage through MII, to reach a similar level to in vivo matured oocytes. However, this augmentation does not have beneficial effect on maturation, fertilization, and early developmental competencies of equine oocytes.

The biosynthesis of GSH is strictly dependent on the availability of cysteine in extracellular compartment (Meister, 1983). However, cysteine is rapidly oxidized in cystine, thus the presence of other thiols, as cysteamine, can maintain cysteine in its reduced form and promote its cellular availability (Meister and Tate, 1976). The addition in the maturation medium of thiols in species other than horse, resulted in an increase in intracellular GSH content and in an improvement of embryonic development (de Matos et al., 1995, 1996, 2002a,b; de Matos et al., 2003; Gasparrini et al., 2003). In our study, a concentration 100 μM of cysteamine was used because this has been demonstrated in being the effective concentration in several mammals (Kito and Bavister, 1997; Gasparrini et al., 2000; Bogliolo et al., 2001; Bogliolo et al., 2002; Rodriguez-Gonzalez et al., 2003; Accardo et al., 2004; Whitaker and Knight, 2004; Kobayashi et al., 2005).

In the present study, the addition of cysteamine affects neither the GSH content in MII oocytes nor the maturation rate of equine oocytes. However, in absence of serum and in presence of BSA as protein source in IVM media, the GSH content was significantly higher than all other maturation conditions tested. We hypothesized that this fact may be due to factor(s) that are present in the serum that can inhibit GSH synthesis. Moreover, the presence of FSH in the maturation medium induced GSH content higher than all the other treatments adopted. However, this high GSH concentration lacks to produce a positive effect on maturation and developmental capability of equine oocyte. On the other hand, our data further support previous studies in which the presence of gonadotropins has been hypothesized to positively regulate ovarian glutathione synthesis through the modulation of glutamate-cysteine ligase enzyme activity, which regulates the first and rate-limiting step of GSH synthesis (Luderer et al., 2001).

From the present data, in vitro development after fertilization of equine oocytes still remains unsuccessful either when matured in presence or in absence of

### Table 3. Effect of Cysteamine Administration on Maturation Rate of Equine Oocytes and Early Embryonic Development After ICSI

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cysteamine</th>
<th>Total MII (%)</th>
<th>Injected &gt;2 cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dMM* + FSH</td>
<td>-</td>
<td>67</td>
<td>46 (68.6)</td>
</tr>
<tr>
<td>dMM + FSH</td>
<td>+</td>
<td>64</td>
<td>47 (73.4)</td>
</tr>
</tbody>
</table>

* dMM is composed of bMM, EGF, and BSA.

**Fig. 1.** GPX (A) and GAPDH (B) gene expression in equine cumulus cells and oocytes. **Lanes 1–3** represent cumulus cells isolated from GV (lane 1) and MII stage oocytes after in vivo collection (lane 2), and expanded cumulus cells after in vitro maturation (lane 3). **Lanes 4–6** represents GV (lane 4) and MII stage (lane 5) oocytes at collection and MII stage after in vitro maturation (lane 6). Semi-quantitative analysis of GPX expression was performed by scanning each band, which was normalized to the intensity of the GAPDH band for the corresponding sample. **Lane N**, negative control of PCR (no cDNA); **lane P**, positive control of PCR; **lane M**, molecular weight markers (100 bp DNA ladder). Values in histograms are mean ± SEM of 5 to 10 samples as indicated in brackets.
cysteamine. Indeed, after ICSI a similar proportion of zygotes in each group developed beyond the two-cell stage after 72 hr of culture, demonstrating that the developmental capability was not affected by the oocyte GSH content.

Our data demonstrate that in equine, cumulus cells expressed GPX mRNA, while GPX transcript was absent from both immature and mature oocytes. Moreover, cumulus expression of GPX mRNA was significantly higher when analyzed at collection than after IVM. While this can be explained by nonphysiological conditions due to in vitro culture, the absence of GPX transcripts in oocyte is in contrast to the findings in human and mouse female gamete (El Mouatassim et al., 1999). In mammals, oxidative stress interferes severely gamete viability and embryo development. A series of antioxidant enzymes and nonenzymatic processes protects gametes and embryos against ROS damage during oocyte maturation and early stage of development (Guerin et al., 1999). For enzymatic defense mechanisms, specific enzymes or their transcripts were identified in follicular fluid (Carbone et al., 2003), oocyte (El Mouatassim et al., 1999; Lonergan et al., 2003a), and oviduct (Gardiner et al., 1998; El Mouatassim et al., 2000; Lapointe and Bilodeau, 2003). In fact, the enzymatic defense against H2O2 includes GPX and classic catalase, both challenged in GSH consumption. While GPX transcript is detected in mouse and human MII oocyte, catalase mRNAs seem to be absent from mature oocyte but is present in mouse blastocyst (El Mouatassim et al., 1999). However, catalase activity has been demonstrated in bovine, porcine, and human oviductal fluids (Lapointe et al., 1998). Moreover, catalase binds to spermatozoa suggesting a potential survival function of female reproductive tract where antioxidant enzymes could bind to gametes and embryos (Lapointe et al., 1998).

Based on studies of Gardiner and Reed (1994, 1995) in murine species, GSH content declines continuously following resumption of meiosis and is approximately 10-fold less by the blastocyst stage of development. The precipitous decline in GSH content is coincident with the increased thermosensitivity of the two-cell embryonic stage (reviewed in Edwards et al., 2001). Embryo does not acquire the ability to synthesize GSH until the blastocyst stage. Further investigations are needed in order to clarify the mechanisms by which equine oocytes-embryos utilize GSH, and defend themselves from oxidative stress through the activation of protective biochemical mechanisms during late stages of oocyte differentiation and early stage of embryonic development. This is particularly important for gene transcripts and polypeptides stored in the oocytes, which sustain early mammalian development (Lonergan et al., 2003b), during the initial transcriptional events in zygote two-cell embryo (Memili and First, 1999) and finally with the major burst of transcription during maternal-embryonic transition (Memili et al., 1998). The study of specific mRNAs and/or translational products involved in gametes and conceptus defense against ROS in equine reproductive tract could bring to better understand the sequential activation of enzymatic defense systems, to ameliorate techniques and protocols for gametes and embryo manipulation and leading to a general improvement of the ART in this species. Taken together, our results demonstrate that in equine oocytes, GSH increases during IVM but the relative intra-oocyte content of this thiol does not affect maturation and early development efficiency after fertilization. We conclude that the limited in vitro developmental competence of equine oocyte is not due to the lack or defective GSH synthesis but to other factor(s) that could be involved in cytoplasmic maturation, oocyte–sperm interaction and early embryonic development.

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