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Glutathione (GSH) Concentrations Vary With the Cell Cycle in Maturing Hamster Oocytes, Zygotes, and Pre-Implantation Stage Embryos

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ABSTRACT Glutathione (GSH) is thought to play critical roles in oocyte function including spindle maintenance and provision of reducing power needed to initiate sperm chromatin decondensation. Previous observations that GSH concentrations are higher in mature than immature oocytes and decline after fertilization, suggest that GSH synthesis may be associated with cell cycle events. To explore this possibility, we measured the concentrations of GSH in Golden Hamster oocytes and zygotes at specific stages of oocyte maturation and at intervals during the first complete embryonic cell cycle. Between 2 and 4 hr after the hormonal induction of oocyte maturation, GSH concentrations increased significantly (~doubling) in both oocytes and their associated cumulus cells. This increase was concurrent with germinal vesicle breakdown and the condensation of metaphase I chromosomes in the oocyte. GSH remained high in ovulated, metaphase II (MII) oocytes, but then declined significantly, by about 50%, shortly after fertilization, as the zygote progressed back into interphase (the pronucleus stage). GSH concentrations then plummeted by the two-cell embryo stage and remained at only 10% of those in MII oocytes throughout pre-implantation development. These results demonstrate that oocyte GSH concentrations fluctuate with the cell cycle, being highest during meiotic metaphase, the critical period for spindle growth and development and for sperm chromatin remodeling. These observations raise the possibility that GSH synthesis in maturing oocytes is regulated by gonadotropins, and suggest that GSH is more important during fertilization than during pre-implantation embryo development. *Mol. Reprod. Dev. 64: 106–112, 2003.* © 2003 Wiley-Liss, Inc.

Key Words: oocyte maturation; glutathione; meiosis; cell cycle; hamster

INTRODUCTION

Glutathione (gamma-glutamylcysteinylglycine, GSH) is a ubiquitous intracellular free thiol that functions in many biological processes including DNA and protein synthesis, drug and chemical metabolism, and cellular protection during oxidative stress (Meister and Anderson, 1983). In addition, GSH and the redox state of the cell also have unique functions in reproduction and early development. GSH concentrations in mature metaphase II (MII) mammalian oocytes are higher than those found in most other tissues (e.g., 8–10 mM in mouse and hamster oocytes); comparable only to concentrations found in hepatocytes (Meister and Anderson, 1983; Calvin et al., 1986; Perreault et al., 1988; Gardiner and Reed, 1994; Furnus et al., 1998). MII oocytes contain more GSH than either immature, germinal vesicle stage oocytes (GV oocytes) or fertilized, pronuclear zygotes in mouse (Calvin et al., 1986); hamster (Perreault et al., 1988); pig (Yoshida et al., 1993; Funahashi et al., 1994); and bovine (de Matos et al., 1997).

Previous work has demonstrated that GSH concentrations are significantly higher in ovulated MII oocytes than in immature GV stage oocytes (Calvin et al., 1986; Perreault et al., 1988). These relatively high concentrations of GSH in mature oocytes appear to play an important role in formation of the male pronucleus after fertilization in the mouse (Calvin et al., 1986), hamster (Perreault et al., 1988), pig (Yoshida, 1993), and cow (Sutovsky and Schatten, 1997). For example, supplementing oocyte maturation culture medium with compounds that promote GSH synthesis (e.g., cysteamine) increased GSH concentrations within cumulus cell-enclosed oocytes and improved subsequent fertilization and embryo development rates during in vitro production of porcine and bovine embryos (Abeydeera et al., 1998; de Matos and Furnus, 2000). Furthermore, oocyte GSH appears to be important for maintaining the meiotic spindle since addition of oxidants to oocytes damages the spindle and results in abnormal zygote formation (Zuelke et al., 1997). That GSH is high when the oocyte is arrested at metaphase, and low when

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the oocyte is in interphase (GV or pronucleus stages), indicates that GSH fluctuates during the meiotic cell cycle, and suggests that the synthesis of GSH may be regulated by the same factors that regulate the cell cycle. However, this possibility has not been examined.

Rather, the relatively high concentrations of GSH in MII stage oocytes have been used as an indicator of oocyte maturation and have been viewed as a potential storage pool of GSH for pre-implantation stages of embryo development (Gardiner and Reed, 1994; Furnus et al., 1998; de Matos and Furnus, 2000). In vivo, the GSH concentrations in mouse embryos drop approximately tenfold from the pronuclear to the blastocyst stages (Gardiner and Reed, 1994). Indeed, in vitro culture conditions that maintain embryonic GSH concentrations at concentrations comparable to those in MII oocytes appear to have a slightly beneficial effect on development to the blastocyst stage in both pigs and cattle (Abeydeera et al., 1998; Furnus et al., 1998; de Matos and Furnus, 2000). To date, however, little is known about the regulation of GSH synthesis during oocyte maturation or its possible association with cell cycle events or factors.

Accordingly, we hypothesized that oocyte GSH concentrations change in close correlation with specific cell cycle stages during oocyte maturation and fertilization. Because cumulus cells interact metabolically with the oocyte during maturation (Buccione et al., 1990; Zuelke and Brackett, 1992, 1993), and since cumulus cells supported maximal GSH synthesis in bovine and porcine oocytes during in vitro maturation (de Matos et al., 1997; Yamauchi and Nagai, 1999), we also hypothesized that cumulus cell GSH concentrations would also change during oocyte maturation. We tested these hypotheses by defining the time-course during which GSH concentrations changed within the oocyte and its associated cumulus cells during in vivo meiotic maturation, and correlated these changes to specific cell cycle stages. We also monitored GSH concentrations after fertilization when the zygote progresses back to interphase (the pronucleus stage). To establish a future benchmark for evaluating potential roles for GSH during fertilization and early embryonic development, we also measured GSH in embryos throughout pre-implantation development.

MATERIALS AND METHODS

Animals, Chemicals, and Culture Medium

Adult (90 days of age) Syrian hamsters (*Mesocricetus auratus*) were purchased from SASCO (Omaha, NE) and acclimatized to a 14–10 hr light–dark period (lights on at 0800 EST) for at least 2 weeks prior to use. Animals were housed individually in polycarbonate cages containing heat-treated pine shavings and given food (Wayne Lab Blocks, Richmond, IN) and tap water ad libitum in a room with ambient temperatures of 20—24°C and relative humidity of 40–50%. Virgin females were examined each morning for 4 days to identify the day of post estrous vaginal discharge and thereby monitor their estrous cycles (Magalhaes, 1970). Animals were killed by cervical dislocation and pneumothorax under nembutal anesthesia.

Reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. Metaphosphoric acid was obtained from Aldrich Chemical Co. (Milwaukee, WI), and Alexa Fluor 488 goat anti-mouse IgG was purchased from Molecular Probes (Eugene, OR). The oocyte/embryo collection medium used in all experiments was phenol red-free, HEPES-buffered Tyrode's medium with amino acids (Perreault and Jeffay, 1993) in which the BSA was substituted with polyvinyl alcohol (10 mg/ml; PVA-Tyrode's) to eliminate extraneous protein contamination during subsequent assay of cumulus cell protein (Zuelke and Brackett, 1992).

Collection of Oocytes, Cumulus Cells, Zygotes, and Embryos for GSH Assay

Oocyte maturation was induced in equine chorionic gonadotropin-primed hamsters (eCG, 20 IU, ip, Diosynth, Chicago, IL) with a single injection of human chorionic gonadotropin (hCG, 25 IU, ip) given 48 hr later. At 0, 2, 4, and 6 hr post-hCG, cumulus cell-enclosed oocytes were obtained via ovarian follicular rupture, washed four times in PVA-Tyrode's, and mechanically denuded of cumulus cells (Perreault and Jeffay, 1993). Oocytes and cumulus cells from individual animals (two animals per time-point) were separated from one another, placed into separate droplets of medium (5 μl/droplet), and prepared for GSH analysis. All oocytes and cumulus cells from each female were combined in a respective single tube for each cell type. Each experimental replicate consisted of two to four groups of oocytes and cumulus cells per treatment (i.e., hours post-hCG) and was repeated at least three times. Ovulated (i.e., MII) cumulus cell-enclosed oocytes were recovered from the oviduct at 16 hr post-hCG, separated from their cumulus cells in PVA-Tyrode's containing hyaluronidase (1 mg/ml), washed four times, and prepared for GSH analysis. Cumulus cells from ovulated oocytes were not assayed for GSH.

To obtain zygotes and pre-implantation embryos, female hamsters were injected with eCG as above, and bred 3 days later. Zygotes were flushed from the oviducts at 8–9 AM on estrus when pronuclei appear small and are in G1 of the first cell cycle, and at 1–3 PM when pronuclei appear full sized and are in G2 (Naish et al., 1987). Embryos were similarly recovered the second morning after breeding (two-cell stage), the second afternoon after breeding (four-cell cell stage), and the fourth morning after breeding (blastocyst stage).

At each time point after hCG, a group of oocytes or zygotes was fixed and stained with acetolacmoid, as described by Perreault and Jeffay (1993), to confirm that they were at the expected developmental stage. To resolve the meiotic cell cycle progression in more detail with respect to chromosome condensation and spindle formation, we visualized these structures in a sample of oocytes selected at random at each time point using

confocal laser scanning microscopy. As described in detail by Barnett et al. (1997), we stained DNA with DAPI and the spindle with anti-β-tubulin (Sigma) as the primary antibody and Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes) as the secondary antibody. A stack or Z series of ~50 images (about 1.5 microns apart) was obtained using a Leica TCS-SP1 confocal laser-scanning microscope (Heidelberg, Germany) equipped with a 63 × water immersion objective (1.2 NA). The Leica software was used to acquire the images (version 2.0 Build 0477) and Imaris (Bitplane, Zurich, Switzerland) software was used to process the images and produce a maximum projection image. To minimize signal overlap between the UV and visible fluorescent lines, the images were sequentially scanned, first with the visible line (excited at 568, emission of 580–630) and then with the UV line (excited at 365, emission of 440–480).

GSH Assay

To prepare samples for GSH analysis, individual media droplets (5 μl) containing either denuded oocytes (25–30 per group), or cumulus cells (a representative sample of the total number of cells obtained from each female equivalent to about 10–15 cumulus complexes per group), zygotes (25–30 per group), or preimplantation stage embryos (40–50 per group), were frozen on dry ice, thawed, and extracted with 5 μl metaphosphoric acid (1.25 M), refrozen, and stored at −60°C as described previously (Calvin et al., 1986; Perreault et al., 1988). Cell-free media blanks were similarly prepared.

Frozen droplets containing the extracted cells or blanks were thawed and centrifuged (14,000 rpm, 2 min) at room temperature to pellet precipitated cellular material. The supernatant was assayed for GSH content as described previously (Tietze, 1969; Brehe and Burch, 1976; Perreault et al., 1988). The tubes containing the remaining cumulus cellular precipitate were kept on ice and later assayed for protein content (see below). The amount of GSH in each sample was determined by comparison with a standard curve prepared at the same time; sensitivity of this GSH assay was approximately 0.125 nmoles of GSH in a final assay volume of 300 μl. This amount was divided by the number of oocytes, embryos, or micrograms of cellular protein in the sample and the result expressed either as picomoles GSH per oocyte (or embryo) or per microgram protein.

Protein Assay

The cellular precipitate remaining from the GSH assay was solubilized on ice with 20 μl 0.5 N NaOH for 30 min. Each 20-μl aliquot was then pipetted into a separate well of a 96-well microtiter plate and assayed for protein content as previously described (Zuelke and Brackett, 1992). A standard curve was generated for each experiment by assaying 20-μl aliquots of BSA standards in 0.5 N NaOH. The lower limit of sensitivity of this assay was between 0.1 and 0.2 μg protein/well; values below 0.2 μg/well were excluded from further analysis.

Data Analysis

Oocytes and cumulus cells from each animal were kept in separate tubes and were assayed at the same time. Analysis of variance indicated that there was no difference in oocyte, or cumulus cell, GSH concentrations between animals or between replicate experiments within each treatment group. Therefore, data from all replicate experiments were combined for final analysis. GSH concentration data (mean ± SEM) were compared using one-way analysis of variance, followed by post-hoc analysis using Fischer's protected least significant difference. Data are expressed as the (mean ± SD) pmol GSH per oocyte (or embryo). Differences where $P < 0.05$ were taken as significant.

RESULTS

GSH Concentrations in Oocytes and Cumulus Cells During Oocyte Maturation

Oocyte GSH concentrations increased significantly from about 1 pmol/oocyte at 0 and 2 hr post-hCG to 1.62 ± 0.16 pmol/oocyte at 4 hr post-hCG (Fig. 1). Confocal laser scanning microscopy allowed us to resolve details of early stages of meiotic maturation manifested as chromosome condensation and spindle formation. These images confirmed that the significant increase in oocyte GSH occurred during the transition from a nucleolar GV (Fig. 2a) or fibrillar GV (Fig. 2b) to prometaphase I when the chromosomes have condensed and the spindle is beginning to form (Fig. 2c,d). Thereafter, GSH concentrations remained elevated in oocytes harvested at metaphase I (6 hr post-hCG; Fig. 2e) and MII (16 hr post-hCG; Fig. 2f).

Cumulus cell GSH concentrations following hCG administration are represented as pmol GSH/μg cell protein (mean ± SEM) from 17, 9, 12, and 6 separate groups of cumulus cells obtained at 0, 2, 4, and 6 hr post-hCG, respectively (Fig. 3). Cumulus cell GSH concentrations increased twofold from about 36 pmol/μg

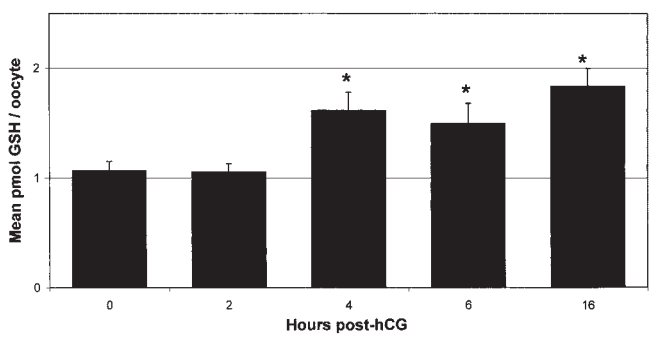

Fig. 1. Changes in hamster oocyte glutathione (GSH) concentrations following induction of in vivo meiotic maturation with hCG. Represented are pmol GSH/oocyte (mean ± SEM) from 22, 12, 11, 12, and 11 separate groups of oocytes collected at 0, 2, 4, 6, and 16 hr post-hCG, respectively. Respective means were compared by ANOVA to that at 0 hr post-hCG; *($P < 0.05$).

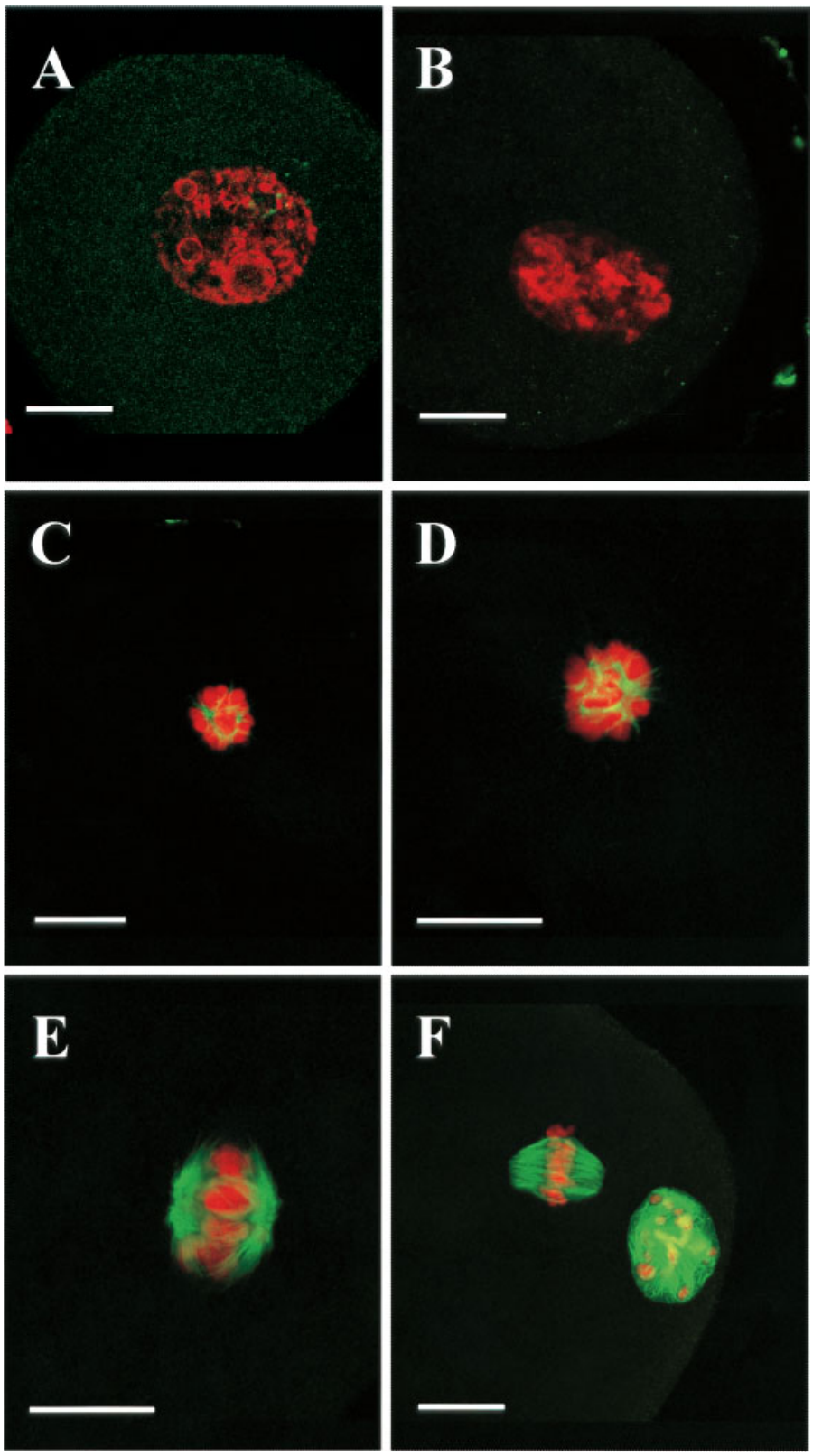

Fig. 2. Confocal laser scanning micrographs of representative hamster oocytes stained to visualize chromatin (red) and spindle microtubules (green) during nuclear maturation at 0, 2, 4, 6, and 16 hr post-hCG. (**A**; 0 hr) Germinal vesicle with well defined nuclear envelope and large nucleoli in an oocyte; (**B**; 2 hr) germinal vesicle (GV) exhibiting early chromatin condensation and decreasing nucleolar organization; (**C** and **D**; 4 hr) condensed MI stage chromosomes and an early meiotic spindle; (**E**; 6 hr) well-formed MI chromosomes and meiotic spindle; (**F**; 16 hr) MII chromosome and spindle and expelled first polar body (to the right of the spindle). Bar = 20 microns.

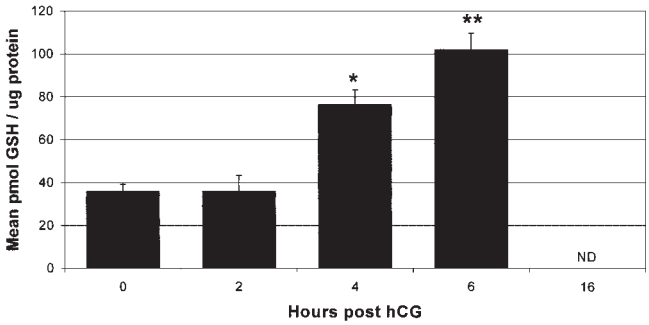

Fig. 3. Changes in hamster cumulus cell GSH concentrations (pmol GSH/μg protein; mean ± SEM) following in vivo administration of hCG. Denoted are significant differences between respective means compared by ANOVA to that at 0 hr post-hCG; *($P < 0.05$); and also between 4 and 6 hr post-hCG; **($P < 0.05$).

protein at 0 and 2 hr post-hCG to 77 pmol/μg protein at 4 hr post-hCG, and increased further to 102 pmol/μg protein by 6 hr post-hCG. This increase in GSH corresponded in time with cumulus expansion that was first observed at 4 hr and became more pronounced by 6 hr post-hCG.

GSH Concentrations in Oocytes, Zygotes, and Pre-Implantation Embryos

The GSH concentrations in hamster oocytes, zygotes, and pre-implantation stage embryos are presented in Table 1. In this series of experiments, GSH concentrations were maximal (3.24 ± 0.49 pmol/oocyte) in MII oocytes and then dropped by about 20% by the early (G1) pronuclear stage, and by another 30% by the late (G2) pronuclear stage. This drop mirrored the rise seen from the GV stage to meiotic MII. GSH concentrations declined further by the two-cell stage and remained at $< 10\%$ of those observed in MII oocytes through the blastocyst stage of development.

DISCUSSION

GSH concentrations increased significantly in both the oocyte and its associated cumulus cells during in vivo meiotic maturation. In both cell types, this increase was concurrent with the resumption of meiosis (i.e., GV breakdown) and formation of MI chromosomes in the oocyte. As reported previously (Calvin et al., 1986; Perreault et al., 1988; Yoshida et al., 1992; Funahashi et al., 1994), mature, MII oocytes typically exhibit maximal concentrations of GSH, in the range of 2–3 pmol/oocyte, or about twice the level observed in GV oocytes. The present results extend these previous observations by showing that GSH concentrations increase significantly between 2 hr post-hCG, when the GV is still intact, and 4 hr post-hCG, when the meiotic chromosomes have condensed. The changes we observed in cumulus cell GSH concentrations were not unexpected since the metabolic interactions between these cumulus cells and oocytes are well documented (Buccione et al., 1990; Zuelke and Brackett, 1992, 1993; Mori et al., 2000). A similar concomitant increase in GSH concentrations has also been reported in cumulus cell-enclosed bovine (Furnus et al., 1998) and porcine (Yamauchi and Nagai, 1999) oocytes. However, since metabolic gap junctions between hamster oocytes and cumulus cells are typically lost beginning around 4 hr post-hCG (Racowsky and Satterlie, 1985), the extent to which GSH metabolism in these two-cell types may be linked or occur independently in response to hCG remains to be determined. The mechanistic implications of this rapid increase in GSH during the early stages of oocyte meiosis are not yet known. The 2-hr interval between administration of hCG and the increase in GSH would provide sufficient time for transcription and subsequent translation of mRNA for gamma-glutamylcysteine synthetase (GCS), the rate-limiting enzyme for GSH synthesis (Soltaninassab et al., 2000), before the maternal chromosomes condensed. However, we did not evaluate these molecular events in this study.

The increase in oocyte and cumulus cell GSH concentrations that occurred shortly after the animals received hCG suggests that GSH metabolism within the ovarian follicle may be under hormonal regulation. Thus, the present data lend support to earlier reports that gonadotropins may regulate ovarian GSH metabolism (Aten et al., 1992; Clague et al., 1992), and indicate that cumulus cell-enclosed oocytes may contribute to the ovarian GSH pool, especially if GSH increases in all of the granulosa cells (not just the cumulus) within affected follicles. Based on these results, we attempted

TABLE 1. Ontogeny of Glutathione (GSH) Concentrations in Hamster Oocytes, Zygotes, and Pre-Implantation Stage Embryos

Stage of development	GSH concentration (mean pmol/oocyte (or embryo) ± SD)	Coefficient of variance (%)	n	% of metaphase II
Metaphase II	3.24 ± 0.49^{a}	15.1	5	100
Early PN	2.61 ± 0.55^{b}	21.1	7	80.6
2 PN	1.71 ± 0.23^{c}	13.5	6	52.8
Two-cell	0.21 ± 0.02^{d}	10.4	5	6.4
Four-cell	0.25 ± 0.03^{d}	13.1	4	7.5
Blastocyst	0.29 ± 0.03^{d}	10.4	5	9.0

n, number of separate groups of oocytes, zygotes, or embryos that were assayed at each stage of development. Values with different superscripts differ from each other, $P < 0.05$ by ANOVA.

to use the hamster cumulus cell-enclosed oocyte as an in vitro model to investigate hormonally regulated GSH metabolism. However, in our hands, hamster oocytes matured in vitro, with or without prior hCG priming, did not synthesize GSH as well as oocytes matured in vivo (data not shown). Perhaps a model system where in vitro oocyte maturation is more established (e.g., porcine or bovine) would be better suited to investigate the important question of whether GSH concentrations in cumulus cell-enclosed oocytes are hormonally regulated during oocyte maturation.

Events occurring during oocyte maturation can significantly impact subsequent oocyte fertilization and embryo development (Zuelke and Brackett, 1990; Yoshida et al., 1992; Eppig, 1996; Krisher and Bavister, 1998). For example, improved embryo development to the blastocyst stage has been reported following in vitro oocyte maturation in media that support increased oocyte GSH concentrations (Abeydeera et al., 1998; De Matos and Furnus, 2000). Formation of a male pronucleus after fertilization has been linked to GSH concentrations within mature, MII oocytes (Calvin et al., 1986; Perreault et al., 1988; Yoshida et al., 1993; Funahashi et al., 1994). The reduced form of GSH has also been implicated in maintaining microtubule function and meiotic spindle morphology during fertilization of hamster oocytes (Zuelke et al., 1997). The close correlation of GSH to meiotic resumption in the oocyte combined with its functional significance during oocyte maturation and embryonic development make it a highly relevant cellular marker of cytoplasmic maturation in mammalian oocytes.

The relatively high concentrations of GSH in MII stage oocytes have been implicated as a potential storage pool of GSH for pre-implantation stages of embryo development (Gardiner and Reed, 1994; Furnus et al., 1998; de Matos and Furnus, 2000). The present results in the hamster indicate that GSH concentrations drop precipitously from MII concentrations as early as the late pronuclear stage zygote and are then maintained at $< 10\%$ of MII concentrations from the two-cell embryo stage through the blastocyst stage of pre-implantation embryo development. A comparable decrease in GSH has been reported in mouse embryos during in vivo fertilization and development (Gardiner and Reed, 1994). The mouse and hamster data together suggest that, in vivo, high concentrations of GSH are needed for oocyte maturation and fertilization, but that after fertilization, GSH is not stored or synthesized to any great extent in the embryo during subsequent pre-implantation development.

CONCLUSIONS

We conclude that oocyte GSH concentrations increase rapidly during a relatively early stage of meiotic maturation and that this increase is closely associated with the nuclear progression of the meiotic cell cycle. Thus, GSH is potentially a highly relevant cellular marker of key regulatory events that may link both cytoplasmic and nuclear oocyte maturation. Since inadequate GSH synthesis or GSH concentrations within pre-implantation embryos have been implicated as potential causes of impaired development during in vitro culture (Takahash et al., 1993; Gardiner and Reed, 1994; Johnson and Nasr-Esfahani, 1994; Lim et al., 1996; Luvoni et al., 1996; De Matos and Furnus, 2000), the mechanisms that regulate GSH and oxidative stress during fertilization and pre-implantation stage embryo development in mammals warrant further study (Stover et al., 2000; Harvey et al., 2002). The present description of the dynamics of GSH concentrations during hamster oocyte maturation and embryonic development provides a comprehensive benchmark for such future studies.

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