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Effects of the addition of glutathione during maturation on *in vitro* fertilisation of prepubertal goat oocytes

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Summary

Glutathione (γ-glutamyl-cysteinyl-glycine; GSH) is a ubiquitous intracellular free thiol that improves development of the male pronucleus at fertilisation and has also been implicated in promoting the development of preimplantation embryos. The objective of this study was to evaluate the effects of adding GSH or cysteine to the *in vitro* maturation medium on intracellular GSH amounts after *in vitro* maturation and fertilisation of prepubertal goat oocytes. Oocytes were matured in TCM199 medium supplemented with 10% bovine fetal serum, 1 mg/ml 17β-estradiol, 10 μg/ml o-FSH, 10 μg/ml LH and 50 mg/ml gentamicin. *In vitro* maturation medium was completed with two independent treatments: GSH at different concentrations (0, 0.25, 0.50 and 1.00 mM) and L-cysteine at different concentrations (0, 150, 300, 600 and 900 μM). After 27 h of culture at 38.5 °C in 5% CO_2 in air, the nuclear stage was evaluated. Simultaneously, another sample of oocytes was frozen and the intracellular GSH level was evaluated with spectrophotometric methodology. Oocytes were inseminated with fresh semen ($2–3 \times 10^6$ sperm/ml) in TALP medium supplemented with 1 mg/ml hypotaurine. Oocytes were fixed at 20 h post-insemination to evaluate the *in vitro* fertilisation. Oocytes matured in 1.00 mM GSH-supplemented medium exhibited higher amounts of intracellular GSH (3.23 pmol per oocyte). The percentage of normal fertilisation (17–27%) was similar for the treatment groups. In conclusion, the addition of 1.00 mM GSH to the maturation medium could be a useful method for increasing the intracellular GSH levels of prepubertal goat oocytes. However, this increase was not associated with a higher normal fertilisation rate of prepubertal goat oocytes.

Keywords: Cysteine, Glutathione, Goat, Oocytes, Prepubertal

Introduction

In vitro matured (IVM) prepubertal goat oocytes often show deficiencies after their *in vitro* fertilisation (IVF), as indicated by a low incidence of male pronuclear formation, first cleavage division, and low developmental competence to blastocyst (Mogas *et al.*, 1997; Izquierdo *et al.*, 1999).

During fertilisation, the sperm nucleus decondenses and is transformed into a male pronucleus (MPN). The mechanisms by which these events take place in the egg cytoplasm are largely unknown. The transformation of the sperm nucleus during IVF has been shown to be related to the levels of intracellular glutathione (GSH). GSH participates in sperm decondensation and in the transformation of the fertilising sperm head into the male pronucleus (Perrault *et al.*, 1988; Calvin *et al.*, 1986; Yoshida *et al.*, 1992; Yoshida, 1993). Consistently, the decrease in intracellular GSH of mouse oocytes impairs the decondensation of the sperm nucleus (Gardiner & Reed, 1994). Therefore, the ability of oocytes to induce the nuclear decondensation of spermatozoa seems to be directly related to high levels of intracellular GSH.

The tripeptide glutathione (γ-glutamyl-cysteinyl-glycine), the major non-protein sulphydryl compound in mammalian cells, is an endogenous and ubiquitous reducing agent that protects cells from oxidation and

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plays a number of important roles in cellular metabolism (reviewed by Meister & Anderson, 1983), including an effect on amino acid transport, DNA and protein synthesis, and reduction of disulphides (Lafleur *et al.*, 1994).

Synthesis of GSH during oocyte maturation has been reported in mouse (Calvin *et al.*, 1986), hamster (Perrault *et al.*, 1988), pig (Yoshida *et al.*, 1993) and bovine oocytes (Miyamura *et al.*, 1995; De Matos & Furnus, 1999). During development and maturation of the oocyte in the ovary, GSH content increases as the oocyte approaches the time of ovulation (Perrault *et al.*, 1988). It is also known that the culture conditions employed for IVM of oocytes can increase the levels of intracellular GSH. GSH synthesis during *in vitro* maturation can be stimulated by the addition of low-molecular-weight thiol compounds (mouse and hamster: Gardiner & Reed, 1994; pig: Wang *et al.*, 1997; Sawai *et al.*, 1997; Yoshida *et al.*, 1990, 1993; cattle: De Matos *et al.*, 1995, 1996, 1997; De Matos & Furnus, 1999).

Yoshida *et al.* (1993) reported that the addition of cysteine to the medium enhances the synthesis of GSH in porcine oocytes. Cysteine is a low-molecular-weight thiol compound predecessor of GSH. Intracellular GSH concentrations have been shown to depend on the availability of cysteine; thus it has been reported that the GSH concentration in oocytes cultured in media without cysteine is depleted, reflecting a low rate of GSH synthesis (Yoshida *et al.*, 1993).

The aim of this study was to assess the effect of adding GSH and cysteine to the IVM medium on intracellular GSH contents after IVM, and pronuclear formation of prepubertal goat oocytes.

Materials and methods

Oocyte collection

Ovaries from prepubertal goats, approximately 2 months old, were obtained from a local slaughterhouse and transported at 37 °C in Dulbecco's phosphate-buffered saline (PBS; Sigma) containing 50 μg/ml gentamicin. Within 2 h of slaughter, the ovaries were washed three times in PBS containing gentamicin. Cumulus–oocyte complexes (COCs) were recovered by slicing the ovaries with a surgical blade in a 60 mm culture dish containing HEPES-buffered TCM199 (Sigma), supplemented with 11.1 μg/ml heparin-sodium salt (170 USP/mg; Sigma) and 50 μg/ml gentamicin. Only oocytes with one or more complete layers of unexpanded cumulus cells and an evenly granulated cytoplasm were selected. Oocytes were washed three times in HEPES-buffered TCM199 and randomly distributed among the treatment groups. The whole process took place at a laboratory temperature of 35 °C.

In vitro maturation of oocytes

Groups of 35–40 cumulus-enclosed oocytes were placed in 500 μl of maturation medium in 4-well dishes (Nunc, Roskilde, Denmark) and incubated for 27 h at 38.5 °C in an atmosphere of 5% CO_2 in air with maximum humidity. The maturation medium was TCM199 (Sigma) supplemented with 10% (v/v) fetal bovine serum (GibcoBRL-Life Technologies, Paisley, UK), 10 μg/ml o-LH (supplied by Dr J.F. Beckers, IRSIA Research Unit, University of Liège, Belgium), 10 μg/ml o-FSH (Ovagen, Immuno Chemicals Products, Auckland, New Zealand), 1 μg/ml 17β-estradiol (Sigma) and 50 μg/ml gentamicin.

To examine the effects of glutathione (GSH), the addition of GSH (Sigma) to the maturation medium was adjusted to: 0, 0.25, 0.50 and 1.00 mM GSH. On the other hand, to examine the effects of cysteine, the cysteine (Sigma) concentration of the *in vitro* maturation medium was adjusted to: 0, 150, 300, 600 and 900 μM cysteine. The concentration ranges were selected from the literature (GSH: Yoshida *et al.*, 1993; Luvoni *et al.*, 1996; cysteine: Boquest *et al.*, 1998; De Matos & Furnus, 1999).

Sperm preparation

At the end of the maturation period, oocytes were inseminated with fresh semen. Ejaculates form Malagueño bucks of proven fertility were collected into artificial vaginas and transported within 30 min to the laboratory at 37 °C. Mortility of sperm cells was assessed under an inverted microscope and the motile sperm fraction was separated by swim-up: 70 μl of semen was placed in each of several conical tubes under 2 ml mDM (Brackett & Oliphant, 1975; modified by Younis *et al.*, 1991), and incubated for 45–60 min in a humidified atmosphere of 5% CO_2 in air at 38.5 °C. After incubation, 600 μl from the top of each tube was removed and pooled in a sterile 15 ml centrifuge tube and centrifuged at 200 *g* for 10 min. After discarding the supernatant, the resulting sperm pellet was resuspended 1:1 with mDM medium containing heparin (100 mg/ml heparin-sodium salt). Finally it was incubated for 45–60 min in a humidified atmosphere of 5% CO_2 in air at 38.5 °C (final suspension 84×10^6 sperm/ml, approximately).

In vitro fertilisation

After maturation, groups of 30–35 oocytes were transferred into 100 μl fertilisation microdrops of modified Tyrode's medium (TALP), as described by Parrish *et al.*

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(1986), supplemented with 1 µg/ml hypotaurine (Sigma, H-1384), covered with 5 ml mineral oil. After capacitation, sperm concentration was assessed in a hemocytometer. An aliquot (5 µl) of the sperm suspension was added to the fertilisation microdrops (final concentration: 3×10^6 sperm/ml). Culture was performed for 24 h under a humidified atmosphere of 5% CO_2 in air at 38.5 °C.

Evaluation of the nuclear stage after IVM and IVF

Nuclear stage after 27 h of IVM and 20 h of IVF was evaluated after fixation and staining with 1% Hoechst 33342. After maturation, oocyte development was measured by the evolutive stages of oocytes reaching metaphase II (MII) stage. The nuclear stages were classified as germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), anaphase I to telophase I (A/T) and metaphase II (MII). After fertilisation, oocytes with a sperm head or a pronucleus were considered to be fertilised and were classified in one or three groups: 2PN (female pronucleus and male pronucleus; normal fertilisation), polyspermy (two or more non-decondensed heads or two or more decondensed heads in the cytoplasm) and 1PN or asynchrony with a non-decondensed sperm head (female pronucleus and a non-decondensed sperm head).

Assay of intracellular glutathione levels

Intracellular content of GSH was measured as described by Funahashi *et al.* (1994). Following IVM, cumulus cells were removed completely from the COCs by shaking in citric acid (Sigma). Five microlitres of 0.2 M sodium phosphate containing 10 mM Na_4-EDTA (Sigma, assay buffer) and 5 µl of 1.25 M phosphoric acid (Fluka, Buchs, Switzerland) were added to a 1.5 ml microfuge tube containing 30 oocytes. Samples were stored at 80 °C until use. Concentrations of GSH in the oocytes were determined by the dithionitrobenzonic acid–glutathione disulphide (DTNB-GSSG) reductase-recycling assay (Anderson, 1985). Briefly, 700 µl of assay buffer containing 0.29 mg/ml NADPH (Sigma) and 100 µl of 0.75 mM 5,5′-dithio-bis-(2-nitrobenzonic acid) (Sigma) was mixed with 190 µl of water in the microfuge tube. After warming at room temperature for 15 min, 10 µl of 250 IU/ml glutathione reductase (Sigma) was added to the tube to initiate the reaction. The formation of 5-thio-2-nitrobenzonic acid, which has an absorption peak at 412 nm, was followed continuously with a spectrophotometer at 20 s intervals. Standards (0.01–1.00 nmol) of GSH (Sigma) and a sample blank lacking GSH were also assayed.

Experimental design

Oocytes were *in vitro* matured, as described before, for 27 h. After this period, a sample of oocytes was examined for nuclear maturation stage and the rest of oocytes were processed for GSH assay or for IVF. To determine whether GSH could stimulate intracellular GSH synthesis and therefore increase intracellular GSH levels, immature oocytes and *in vitro* matured oocytes were frozen and the amount of intracellular GSH was evaluated spectrophotometrically. Intracellular GSH measurement were performed in groups of 30 oocytes in each treatment group in all experiments. Replicates of experiments were performed on different days with different batches of oocytes.

Experiment 1

We studied the effect of the addition of GSH to the *in vitro* maturation medium on nuclear stage and intracellular GSH levels after IVM, and IVF parameters of prepubertal goat oocytes. COCs were *in vitro* matured in the presence of 0 (control), 0.25, 0.50 and 1.00 mM GSH.

Experiment 2

We studied the effect of the addition of GSH to the *in vitro* maturation medium on nuclear stage and intracellular GSH levels after IVM, and IVF parameters of prepubertal goat oocytes. COCs were *in vitro* matured in the presence of 0 (control), 150, 300, 600 and µM L-cysteine.

Statistical analysis

The frequencies of nuclear maturation and fertilisation were compared using the Least Square Means method test following an analysis of variance (ANOVA) using Statistical Analysis System program (SAS Institute, Cary, NC, 1987). The frequencies of intracellular GSH levels were subjected to log transformation before statistical analysis. The statistical significance of the results was analysed by one-way ANOVA using the SAS program, and for detailed comparison among the GSH levels the Tukey test was used. A *p* value less than 0.05 was considered to be significant.

Results

Effect of glutathine on nuclear maturation

The nuclear stage of 943 oocytes after IVM was assessed in 8 replicates. Table 1 shows the different rates of nuclear maturation obtained after exposure to different GSH concentrations (0.25, 0.50 and 1.00 mM). Overall, there were no significant differences in

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Table 1 Effect of addition of GSH to the maturation medium on nuclear maturation of prepubertal goat oocytes

Glutathione (mM)	No. of replicates	*n*	GV (%)	GVBD (%)	MI (%)	A/T (%)	MII (%)
Control	8	241	19 (7.88)	1 (0.41)	30 (12.45)	6 (2.49)	185 (76.76)
0.25	8	214	20 (9.35)	1 (0.47)	20 (9.35)	3 (1.40)	170 (79.44)
0.50	8	244	17 (6.97)	1 (0.41)	25 (10.25)	5 (2.05)	196 (80.33)
1.00	8	244	21 (8.61)	1 (0.41)	28 (11.48)	4 (1.64)	190 (77.87)

There were no significant differences among the different groups.
GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphase I; A/T, anaphase/telophase; MII, metaphase II.

nuclear maturation between control and GSH-exposed oocytes, independent of the GSH concentration.

Effect of glutathione on intracellular glutathione levels

The intracellular GSH level of 1320 oocytes cultured in GSH-supplemented maturation medium was assessed in 6–10 replicates. Fig. 1 shows that intracellular GSH level increased ($p < 0.05$) in oocytes when IVM medium was supplemented with 1100 mM GSH (3.23 pmol per oocyte) compared with immature oocytes (1.21 pmol per oocyte). The intracellular GSH level was higher in oocytes matured with GSH than in maturation medium alone; nevertheless these differences were not significant ($p = 0.057$).

Effect of gluthatione on *in vitro* fertilisation

After *in vitro* maturation in a GSH-supplemented medium, 874 oocytes were *in vitro* fertilised in 6 replicates. Very stable penetration rates (35–43%) were obtained for all GSH concentrations in the maturation medium (Table 2). There were no significant differences in the incidence of 2PN oocytes among treatment groups (22–28%). Moreover, the addition of GSH to the maturation medium did not significantly affect ($p > 0.05$) the rate of 1PN, the incidence of polyspermy and the proportion of 2PN oocytes with respect to the total penetrated.

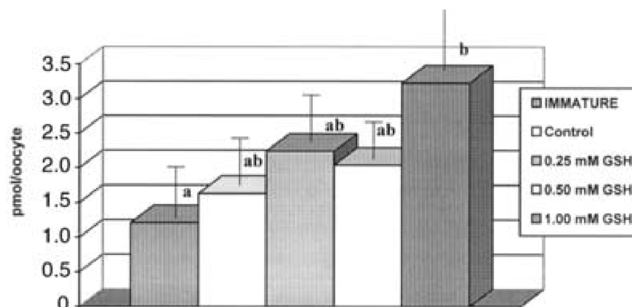

Figure 1 Effect of the addition of GSH to the *in vitro* maturation medium on intracellular GSH levels of prepubertal goat oocytes (pmol per oocyte). [a,b] different superscripts show significant differences ($p < 0.05$).

Effect of cysteine on *in vitro* maturation

The nuclear stage of 762 oocytes after IVM in a cysteine-supplemented medium was assessed in 7 replicates. Table 3 shows the different rates of nuclear maturation obtained after exposure to different L-cysteine concentrations (150, 300, 600 and 900 μM L-cysteine). There were no significant differences in nuclear maturation between control and L-cysteine exposed oocytes, independently of the L-cysteine concentration.

Effect of cysteine on the intracellular glutathione levels

The intracellular GSH level of 1498 oocytes cultured in maturation medium for 27 h was assessed in 7–12 replicates. As shown in Fig. 2, no significant differences

Table 2 Effect of the addition of GSH to the maturation medium on *in vitro* fertilisation of prepubertal goat oocytes

GSH (mM)	No. of replicates	*n*	IVF				
			Penetration (%)	2PN(%)	1PN(%)	Polyspermy (%)	2PN/ penetration %
Control	6	236	83 (35.17)	54 (22.88)	11 (4.66)	18 (7.62)	65.06
0.25	6	230	84 (36.52)	52 (22.60)	16 (6.95)	18 (7.82)	61.90
0.50	5	210	92 (43.81)	47 (22.38)	20 (9.52)	25 (11.90)	51.09
1.00	6	198	83 (41.91)	55 (27.77)	13 (6.56)	15 (7.57)	75.34

There were no significant differences among treatment groups.
IVF, *in vitro* fertilisation; 2PN, oocyte with male and female pronuclei; 1PN, oocyte with only one pronucleus.

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Table 3 Effect of addition of cysteine to the maturation medium on nuclear maturation of prepubertal goat oocytes

Cysteine (µM)	No. of replicates	*n*	GV (%)	GVBD (%)	MI (%)	A/T (%)	MIII (%)
Control	7	156	17 (10.90)	2 (1.28)	21 (13.46)	3 (1.92)	113 (72.44)
150	7	155	11 (7.10)	0 (0.00)	21 (13.55)	8 (5.16)	115 (74.19)
300	7	141	8 (5.67)	1 (0.71)	20 (14.18)	5 (3.55)	107 (75.89)
600	7	158	14 (8.86)	1 (0.63)	18 (11.39)	4 (2.53)	121 (76.58)
900	7	152	11 (7.24)	1 (0.66)	18 (11.84)	2 (1.32)	120 (78.95)

There were no significant differences among the different groups.
GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphase I; A/T anaphase/telophase; MII, metaphase II.

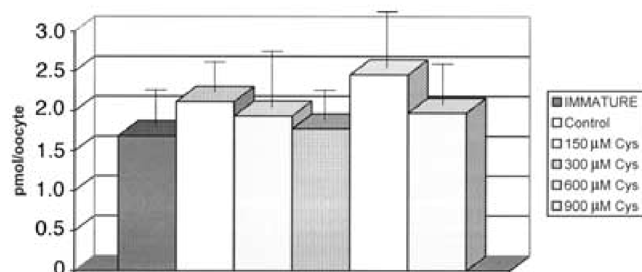

Figure 2 Effect of the addition of cysteine to the *in vitro* maturation medium on the intracellular GSH levels of prepubertal goat oocytes (pmol per oocyte). There were no significant differences among the different groups.

were observed in oocyte GSH levels after IVM when oocytes were cultured in maturation medium with the addition of several cysteine concentrations.

Effect of cysteine on *in vitro* fertilisation

Table 4 shows the *in vitro* fertilisation parameters of 789 *in vitro* matured oocytes. There were no significant differences in the meiotic process between oocytes cultured in the presence or in the absence of cysteine in the maturation medium. Penetration rates (3–8%) were not significantly different for any cysteine concentration in maturation medium. There were no significant differences in the incidence of 2PN oocytes among treatment groups (18–26%). The addition of cysteine to the maturation medium did not significantly ($p > 0.05$) affect the rate of 1PN oocytes, the incidence of polyspermy and the proportion of 2PN oocytes with respect to the total penetrated oocytes.

Discussion

In this study the addition of different thiol compounds (GSH and cysteine) to the maturation medium did not enhance the metaphase II rates of prepubertal goat oocytes. Yoshida *et al.* (1992, 1993) reported that during *in vitro* maturation of porcine oocytes, the depletion of intracellular GSH concentration had no effect on the nuclear maturation ability. Intracellular GSH concentrations in prepubertal goat oocytes before the beginning of the meiotic process are probably just sufficient for effective nuclear maturation. Nuclear maturation is an essential process in oocyte maturation. Nevertheless, nuclear maturation does not ensure the developmental competence of an oocyte. The oocyte requires other changes in cumulus cells, the plasma membrane and cytoplasm in order to allow normal fertilisation. Therefore, the oocyte's ability to support sperm head decondensation and MPN formation depends on cytoplasmic maturation (Szöllösi, 1993). On the other hand, cumulus cells around the hamster oocyte have a high GSH concentration (Zuelke & Perrault, 1994). The physiological requirements of intracellular GSH of prepubertal goat oocytes could be perfectly met by the cumulus mass.

Table 4 Effect of the addition of cysteine to the maturation medium on *in vitro* fertilisation of prepubertal goat oocytes

Cysteine (µM)	No. of replicates	*n*	IVF				
			Penetration (%)	2PN (%)	1PN (%)	Polyspermy (%)	2PN/penetration (%)
Control	5	159	52 (32.70)	28 (17.61)	5 (3.14)	19 (11.95)	53.85
150	5	174	69 (40.35)	35 (20.11)	9 (5.17)	25 (14.36)	50.72
300	4	130	58 (44.62)	24 (18.46)	9 (6.92)	25 (19.23)	41.38
600	5	174	71 (40.80)	42 (24.13)	8 (4.59)	21 (12.07)	59.15
900	5	155	75 (48.39)	40 (25.80)	5 (3.22)	30 (19.35)	53.33

There were no significant differences among treatment groups.
IVF, *in vitro* fertilisation; 2PN, oocyte with male and female pronuclei; 1PN, oocyte with only one pronucleus.

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GSH, the major intracellular free thiol, has important biological functions during cellular proliferation, amino acid transport, and synthesis of protein and DNA (Meister & Anderson, 1983). Thiol compounds are the natural reserves of cellular reducing ability, and therefore are considered as the main provider of the oocyte's ability to bring about sperm head decondensation, and its main defence against the cytotoxic effect of hydrogen peroxide (Meister, 1983). Several investigators have shown that SH is synthesised and accumulated during the maturation process in hamster (Perrault *et al.*, 1988), porcine (Yoshida *et al.*, 1993; Takahashi *et al.*, 1993) and bovine oocytes (Miyamura *et al.*, 1995; De Matos *et al.*, 1995, 1999). In the early maturational stages, the GSH supply is dependent on transport through the plasma membrane. On the other hand, Meister *et al.* (1976) reported that the cell's ability for GSH transport depends on the γ-glutamyl cycle, where cells require splitting up the GSH reassembly ability. Therefore, the inclusion of GSH precursor amino acids (Gln, Cys, Gly) is beneficial for the oocyte's GSH synthesis (Yoshida *et al.*, 1993). In our study we observed that intracellular GSH levels of oocytes matured in media supplemented with 1.00 mM GSH are higher than those in immature oocytes. Thus the addition of high GSH concentrations (1.00 mM) stimulates the increase of intracellular GSH levels. Consequently, the inclusion of high GSH levels is essential for the oocyte's ability for spermatozoa decondensation (Telford *et al.*, 1990; Gardiner & Reed, 1994).

De Matos *et al.* (1997) and Yoshida *et al.* (1993) showed that the addition of cysteine stimulates the intracellular GSH synthesis of bovine denuded oocytes and porcine ooctyes, respectively. It has been reported that GSH synthesis is dependent on the availability of cysteine in the medium (Meister & Tate, 1976; Lafleur *et al.*, 1994). Nevertheless, in the present study we observed that the intracellular GSH content of prepubertal goat oocytes at the end of the *in vitro* maturation period was not increased by the use of cysteine in the culture medium for *in vitro* maturation. Therefore, in spite of being considered a limiting factor for GSH synthesis, cysteine is not the only determinant responsible for GSH synthesis in prepubertal goat oocytes. In this study we have observed that cysteine supplementation of the maturation medium did not afford any beneficial effect on the fertilisation parameters of prepubertal goat oocytes.

Zirkin *et al.* (1989) reported that in mammalian oocytes, the pool of GSH, which is synthesised during the first meiosis, seems to ensure the reduction of the sperm protamines that are responsible for the hypercondensation and packing of the sperm DNA. After a reduction in disulphide bond stabilisation, the protamines are replaced by oocyte-derived histones, and the sperm nucleus develops into the male pronucleus (Zirkin *et al.*, 1989). It is believed that at fertilisation a high intracellular GSH content is needed for the transformation of the disulphide-stabilised sperm nucleus into the male pronucleus in mouse (Calvin *et al.*, 1986), hamster (Perreault *et al.*, 1988) and porcine oocytes (Yoshida *et al.*, 1993; Yoshida, 1993). This transformation includes the decondensation of the sperm head and the dispersion of sperm chromatin in the oocyte cytoplasm (Zirkin *et al.*, 1989). Zirkin *et al.* (1985) and Sutovsky & Schalten (1997) related the amount of intracellular GSH to the prevalence of oocyte penetration, and male and female pronucleus formation. Consequently, the intracellular GSH level could be a good biochemical marker of oocyte stage and a measure of optimum condition of the medium. In the present study we did not observe significant differences in the fertilisation parameters among the experimental groups. Therefore, this observation suggests that another oocyte factor besides GSH, such as male pronucleus growth factor (MPGF; *Crozet*, 1993), may control male pronucleus formation; on the other hand, this is probably insufficient in prepubertal goat oocytes for sperm head decondensation. We have observed that prepubertal goat oocytes cultured in maturation media supplemented with 1.00 mM GSH are able to increase the intracellular amounts of GSH. Nevertheless, we detected no increase in the normal fertilisation parameter rates in oocytes matured in the presence of a maturation medium supplemented with GSH.

Since sperm nuclei with rich disulphide bonds decondense and transform into pronuclei more slowly than disulphide-poor ones (Perrault *et al.*, 1988), and male pronucleus formation is dependent on oocyte activation, sperm decondensation may take place more slowly or incompletely in oocytes containing lower than normal GSH levels. This may result in the asynchronous development of the oocyte and sperm nuclei (Zirkin *et al.*, 1989). It is important to point out that in spite of being an essential factor, intracellular GSH levels are not the only determinant of sperm head decondensing ability. It is logical to assume that sufficient GSH levels are maintained in the oocyte thanks to active GSH regeneration (Perrault & Zirkin, 1982; Perrault *et al.*, 1987). Glutathione is a critical intracellular antioxidant because of its relative abundance and ability to cycle readily between reduced (GSH) and oxidised forms (glutathione disulphide; CSSG). The balance between GSH, the active compound, and GSSG is maintained through rapid recycling of GSSG back to GSH via GSSG reductase and thanks to NADPH as electrons donor (Meister, 1983). Consequently, the primary reductors are NADPH, the product of the pentose phosphate pathway. This metabolic route can be considered as an effective metabolic control of oocyte potential ability (Gardiner & Reed, 1994). However, *in vitro* matured hamster oocytes have

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a limited ability to recover GSH homeostasis (Zuelke *et al.*, 1997). Similarly, prepubertal goat oocytes during *in vitro* maturation possibly do not have sufficient reducing ability to ensure GSH regeneration from the oxidised form, GSSG. Consequently, an impaired pathway could result in the depletion of the reducing power of the cell and the inability to regenerate GSH.

In conclusion, our results indicate that the addition of GSH to the maturation medium at the concentration of 1.00 mM results in increased intracellular GSH levels in *in vivo* matured prepubertal goat oocytes. However, this increase in intracellular GSH levels was not associated with a higher than normal fertilisation rate of prepubertal goat oocytes.

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