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Oocyte glutathione and fertilisation outcome of *Macaca nemestrina* and *Macaca fascicularis* in *in vivo*- and *in vitro*-matured oocytes

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Abstract

Fertilisation and development of IVM non-human primate oocytes is limited compared with that of *in vivo*-matured (IVO) oocytes. The present study describes the IVM of macaque oocytes with reference to oocyte glutathione (GSH). Timing of maturation, comparison of IVM media and cysteamine (CYS) supplementation as a modulator of GSH were investigated. A significantly greater proportion of oocytes reached MII after 30 h compared with 24 h of IVM. Following insemination, IVM oocytes had a significantly lower incidence of normal fertilisation (i.e. 2PN = two pronuclei and at least one polar body) and a higher rate of abnormal fertilisation (1PN = one pronucleus and at least one polar body) compared with IVO oocytes. Immunofluorescence of 1PN zygotes identified incomplete sperm head decondensation and failure of male pronucleus formation as the principal cause of abnormal fertilisation in IVM oocytes. The IVO oocytes had significantly higher GSH content than IVM oocytes. Cumulus-denuded oocytes had significantly lower GSH following IVM compared with immature oocytes at collection. Cysteamine supplementation of the IVM medium significantly increased the GSH level of cumulus-intact oocytes and reduced the incidence of 1PN formation, but did not improve GSH levels of the denuded oocyte. Suboptimal GSH levels in macaque IVM oocytes may be related to reduced fertilisation outcomes.

Keywords

cumulus-denuded oocyte; cysteamine; male pronucleus formation

Introduction

Oocyte maturation involves several complex cellular processes encompassing both nuclear and cytoplasmic maturation. Coordination of these events is critical for the normal maturation of the oocyte and subsequent fertilisation and embryo development. Development of non-human primate (NHP) oocytes matured *in vitro* is inferior to their *in vivo*-matured (IVO) counterparts, primarily due to our poor understanding of the molecular processes involved in cytoplasmic maturation (Schramm *et al.* 2003). A very limited number of IVM NHP oocytes have been used successfully to derive live-born off-spring (Schramm and Paprocki 2000). The

development of IVM procedures that support the production of fully competent oocytes in primates has important implications for assisted reproduction in both NHPs and humans.

The IVM of oocytes derived from unstimulated ovaries provides a unique opportunity to investigate the acquisition of oocyte developmental competence. Although the NHP is an excellent translational model for studying processes related to human reproduction (Schramm and Bavister 1999), currently there is very little information on culture requirements for the IVM of primate oocytes. To date, most work related to NHP IVM has focused on the rhesus macaque (*Macaca mulatta*; Schramm and Bavister 1995; Zheng *et al.* 2001; Schramm *et al.* 2003; VandeVoort *et al.* 2003; Zheng 2007) and specific requirements, such as incubation time and culture media, for other species of laboratory macaque (*Macaca nemestrina* and *Macaca fascicularis*) have yet to be determined.

During oocyte maturation, the γ -glutamyl cycle is responsible for the *de novo* synthesis of intracellular glutathione (GSH). Synthesis of GSH directly affects the acquisition of developmental competence of the mammalian oocyte at the cytoplasmic level (Eppig 1996) and may be a reliable indicator of oocyte viability. GSH is involved in protection against oxidative stress (Deneke and Fanburg 1989) and male pronucleus (MPN) formation coincident with oocyte activation (Yoshida 1993). GSH stores attained during maturation act as a reservoir for the oocyte through fertilisation and transition to embryonic genome activation. The importance of GSH during oocyte maturation has been explored in several non-primate mammalian species (Yoshida 1993; de Matos *et al.* 1996; Zuelke *et al.* 1997; Salmen *et al.* 2005) and has been identified as being of paramount importance for normal fertilisation and improved embryo development to the blastocyst stage following IVM. To date, examination of oocyte GSH content and specific manipulation of the γ -glutamyl cycle during oocyte maturation in the NHP and human has not been undertaken.

The first rate-limiting step in GSH synthesis is the availability of the amino acid cysteine for the enzyme glutamate cysteine ligase (GCL; Griffith and Meister 1979, formerly γ -glutamylcysteine synthetase EC 6.3.2.2). Exogenous cysteine is very unstable and readily oxidises to cystine, making it unavailable to the oocyte within a matter of hours (Ishii and Bannai 1985). Exogenous cystine relies on the X_c⁻ transport system, which is relatively inactive in the maturing oocyte until the MII stage and is likely to be of little benefit to the oocyte during IVM (Pelland *et al.* 2009). IVM studies in a range of non-primate mammalian species have used cysteamine (CYS) supplementation to increase the availability of cysteine (Gruppen *et al.* 1995; de Matos *et al.* 2003; Gasparrini *et al.* 2003; Rodríguez-González *et al.* 2003; Luciano *et al.* 2005). CYS acts as a delivery system by reducing cystine and increasing the intracellular supply of cysteine for GSH synthesis (Issels *et al.* 1988). The specific effects of CYS as a GSH modulator in an NHP IVM setting have not yet been explored.

In the present study, culture conditions were optimised for the IVM of *M. nemestrina* and *M. fascicularis* oocytes collected from unstimulated ovaries. The maturation and fertilisation outcomes of both IVO and IVM oocytes and the relationship between oocyte GSH and fertilisation are described. In addition, we investigated the use of CYS, as a known modulator of GSH in other non-primate mammalian species, and determined its effect for the first time on maturation, GSH content and fertilisation of macaque IVM oocytes.

Materials and methods

Unless indicated otherwise, chemicals were obtained from Sigma Chemical (St Louis, MO, USA). All animal studies were approved by the University of Washington Institutional Animal Care and Use Committee (3387-01). Animals were anaesthetised with a mixture of ketamine (10 mg kg⁻¹, i.m.), propofol (4 mg kg⁻¹, i.v.) and isoflurane given to effect (1.5–4%) in

combination with pre- and post-operative analgesia (7.5 mg kg⁻¹, i.m., ketoprofen and 0.15 mg kg⁻¹, i.m., butorphanol).

Ovarian stimulation and oocyte retrieval

Adult female *M. nemestrina* ($n = 16$) and *M. fascicularis* ($n = 11$) with regular menstrual cycles, aged 6.3–16.5 years and weighing between 2.9 and 12.9 kg were housed at the Washington National Primate Research Center (WaNPRC). The animals were caged individually, with access to regular social contact, under a 12-h light–dark cycle (lights on from 0600 to 1800 hours), with temperature maintained at 22°C. Oocyte donors were subjected to ovarian stimulation with twice daily injections of recombinant human (rh) FSH (60 IU day⁻¹, i.m.; Serono, Rockland, MA, USA), starting 1 day after the beginning of menses and continuing for 11–13 days. Follicular maturation was completed with administration of human chorionic gona-dotrophin (hCG; 1000 IU, i.m.; Serono). Oocytes were retrieved from anaesthetised animals at laparotomy 28–30 h after hCG administration. Follicular aspirate was collected into HEPES-modified human tubal fluid (mHTF; Irvine Scientific, Santa Ana, CA, USA) supplemented with 0.3% w/v bovine serum albumin (BSA). Harvested oocytes were treated with hyaluronidase (40 IU mL⁻¹) to remove cumulus cells and classified according to nuclear maturation status (i.e. germinal vesicle (GV), MI or MII). MII oocytes were transferred to 20-μL drops of fertilisation medium (human tubal fluid (HTF) supplemented with 0.3% w/v BSA) under mineral oil and incubated in a humidified atmosphere of 6% CO₂ in air at 37°C for 1–2 h before insemination.

Oocyte IVM

Macaca nemestrina and *M. fascicularis* ovaries ($n = 47$ pair) were obtained from the WaNPRC tissue redistribution programme and transported to the laboratory at 35°C within 1 h of collection. Cumulus–oocyte complexes (COC) were retrieved by ovarian dissection and follicle puncture into mHTF + BSA at 37°C. Selected oocytes were cultured in 800 μL maturation medium in an organ culture dish (BD Falcon, Franklin Lakes, NJ, USA) in a humidified atmosphere of 6% CO₂ in air at 37°C. Maturation was performed either in modified (i.e. minus polyvinyl alcohol (PVA) and pantothenic acid, plus 1× minimum essential medium (MEM) and 1× basal medium eagle amino acid solutions) hamster embryo culture medium (mHECM-10; Zheng *et al.* 2001) or modified Connaught Medical Research Laboratories medium (mCMRL-1066; Invitrogen, Carlsbad, CA, USA). CMRL-1066 is a complex medium consisting of basic salts and a range of ingredients including glucose, amino acids, vitamins and nucleosides; HECM-10 is a less complex medium comprised of basal salts, glucose and amino acids. Both media were supplemented with L-glutamine (0.4 mM), pyruvate (0.25 mM), sodium lactate (10 mM), gentamycin sulfate (50 μg mL⁻¹), 1.0 IU rhFSH (Serono), 1.0 IU human (h) LH, oestrogen (1 μg mL⁻¹), progesterone (3 μg mL⁻¹) and 10% v/v fetal calf serum (FCS; Hyclone, Waltham, MA, USA). Following maturation, oocytes were treated with hyaluronidase (40 IU mL⁻¹) to remove cumulus cells and classified according to nuclear maturation status (GV, MI or MII).

Oocyte IVF

The MII oocytes were washed twice in mHTF + BSA and once in fertilisation medium (HTF + BSA) before being transferred to individual 20-μL drops of HTF + BSA under mineral oil and inseminated. Frozen epididymal spermatozoa were thawed in a 33°C water bath for 1 min. Motile spermatozoa were prepared by centrifugation (500g) on a single 80% Puresperm density gradient (NidaCom Laboratories, Gothenburg, Sweden) for 10 min at room temperature. The spermatozoa were retrieved and repelleted by centrifugation at 400g for 5 min in mHTF + BSA. Final sperm preparations were capacitated in the presence of 0.5 mM caffeine and 0.1 mM dibutyryl-cAMP at 37°C for 5 min. Hyperactivated motile spermatozoa (0.5×10^6

mL⁻¹) were added to the oocytes and incubated for 12–15 h in a humidified atmosphere of 6% CO₂ in air at 37°C. The moment of addition of the spermatozoa to the oocyte was taken as the time of insemination. Fertilisation was assessed visually by the presence of PN and polar bodies and recorded as 2PN (two pronuclei and at least one polar body), 1PN (one pronucleus and at least one polar body), >2PN (multiple pronuclei and at least one polar body) or FF (failed fertilisation; no pronuclei and one polar body).

GSH sample preparation and analysis

Oocytes subjected to GSH assay were denuded of cumulus cells by manual pipetting with a small-bore glass pipette in 40 IU mL⁻¹ hyaluronidase. Oocytes were washed four times in phosphate-buffered saline (PBS) supplemented with 1 mg mL⁻¹ PVA. Oocytes were transferred in 5 µL PBS + PVA into the bottom of an Eppendorf tube (1–10 oocytes per tube). Preliminary experiments indicated that there is no effect of sample size ($n = 1–20$ oocytes per tube) on the measure of total GSH (data not shown). Samples were snap frozen in liquid nitrogen (LN₂) and stored at –30°C. The total GSH content of oocytes was determined using a commercial 5,5'-dithiobis 2-nitrobenzoic acid (DTNB)–GSH reductase recycling assay kit (NWK-GSH01; Northwest Life Science, Vancouver, WA, USA). Oocytes were thawed and 5 µL of 1.25 M phosphoric acid was added to each tube. Samples were snap frozen in LN₂ and thawed at room temperature a further three times to ensure complete cell lysis. On the final thaw, 40 µL assay buffer was added to each tube. Samples and standards were prepared according to manufacturer's instructions, loaded onto a microtitre plate reader (Titertek, Huntsville, AL, USA) and analysed at 405 nm with repeated reads at 2-min intervals for 30 min. A calibration curve was constructed from the standards and the rate of change of absorbance was determined by linear regression analysis for each sample and blank. Concentrations of total GSH (pmol per oocyte) were calculated from the standard curve. The interassay CV was 11.6% across 16 replicate plates.

Immunofluorescent assessment of fertilisation

At 12–15 h post insemination, oocytes that failed to fertilise (FF) and oocytes with one pronucleus (1PN) were washed three times in protein-free mHTF and fixed in 2% paraformaldehyde + 0.1% Triton X-100 at 37°C for 40 min. Dual staining for tubulin (green) and chromatin (blue) was performed to confirm penetration of spermatozoa in 1PN oocytes (sperm head, blue; tail, green) and the status of female chromatin and the spindle apparatus in FF oocytes. For tubulin and chromatin staining, oocytes were first blocked in PBS supplemented with 4% goat serum (GS) for 30 min at room temperature, then incubated in mouse monoclonal anti- α -tubulin antibody (diluted 1:1000 in PBS + 1.5% GS) at 37°C for 1 h. After two washes in PBS + 1.5% GS (for 15 min each), oocytes were incubated in Alexa Fluor 488-labelled goat anti-mouse secondary antibody (diluted 1:100; Invitrogen, Carlsbad, CA, USA) plus 25 µg mL⁻¹ bisbenzimidazole (Hoechst 33258) at 37°C for 1 h. Oocytes were then washed in PBS + 1.5% GS and mounted in a 50% glycerol solution on a microscope slide and examined using an inverted microscope (Nikon E400; Nikon, Melville, NY, USA) fitted with an ultraviolet lamp and appropriate excitation filters (Hoechst, 330–380 nm; fluorescein isothiocyanate (FITC), 494–518 nm).

Oocytes with 1PN and containing a sperm head within the cytoplasm were considered fertilised (1PN; female pronucleus and a condensed sperm head and at least one polar body). In the absence of sperm penetration, oocytes were either classified as FF (failed fertilisation; metaphase chromatin and one polar body) or SpA (spontaneous activation; one pronucleus and at least one polar body).

Experimental design

Both immature oocytes at collection (GV) and resultant MII oocytes following IVO or IVM are described in the following experiments. For clarification, subgroups of oocytes are described as follows: (1) immature GV oocytes derived from unstimulated ovaries (UnSt-GV); (2) MII oocytes following IVM of GV oocytes derived from unstimulated ovaries (UnSt-MII); (3) immature GV oocytes derived from stimulated ovaries (St-GV); (4) MII oocytes derived from stimulated ovaries (St-MII); or (5) MII oocytes following IVM of GV oocytes collected from stimulated ovaries (St-GV-MII).

Experiment 1: effect of time on maturation and fertilisation of macaque oocytes from unstimulated ovaries

Immature oocytes retrieved from unstimulated ovaries (UnSt-GV; three replicates) were divided into two experimental groups and placed in mHECM-10. Oocytes were matured for either 24 or 30 h. Following maturation, oocytes were denuded of cumulus cells and classified according to nuclear maturation status as described above.

Mature oocytes (UnSt-MII) from the 24 and 30 h IVM groups were subjected to IVF and evaluated for pronuclear formation 12–15 h after insemination as described above. The MII oocytes from an additional three replicates of the 30-h maturation protocol were inseminated and resultant 1PN and FF oocytes prepared for immunofluorescence assessment.

Experiment 2: comparisons of media for maturation and fertilisation of macaque oocytes from unstimulated ovaries

Based on results from Experiment 1, UnSt-GV oocytes were matured for 30 h in all subsequent IVM experiments.

To ascertain the optimal IVM medium for macaque oocytes, the maturation rate and fertilisation outcome between simple (mHECM-10) and complex (mCMRL-1066) media were compared. Immature oocytes collected from unstimulated ovaries (UnSt-GV; eight replicates) were selected and matured in either mHECM-10 or mCMRL-1066 for 30 h. Oocytes were denuded and maturation assessed as either GV, MI or MII. Mature oocytes (UnSt-MII) were subjected to IVF and checked for pronuclear formation 12–15 h after insemination as described above.

Experiment 3: comparison of fertilisation outcome and GSH content of IVO and IVM macaque oocytes

Oocytes were retrieved from both stimulated cycles (St-GV and St-MII; $n = 27$) and unstimulated ovaries (UnSt-GV; $n = 27$ pairs) as described above. *In vivo*-derived oocytes were denuded of cumulus cells and assessed for stage of maturation. Immature St-GV oocytes at the time of collection were processed for GSH assay. Mature St-MII oocytes were allocated to either GSH assay or used for fertilisation studies.

Immature UnSt-GV oocytes from unstimulated ovaries were randomly assigned to GSH assay or IVM in mCMRL-1066. Following maturation, UnSt-MII oocytes were either processed for GSH assay or inseminated.

Experiment 4: maturation, GSH content and fertilisation outcome of COCs and denuded macaque oocytes matured in the presence or absence of CYS

Based on results from Experiment 2, UnSt-GV oocytes were matured in mCMRL-1066 medium for 30 h in all subsequent IVM experiments.

UnSt-GV oocytes were subjected to IVM in mCMRL-1066 as COC (14–16 replicates) or denuded oocytes (DO; eight replicates) in the presence or absence of 100 μ M CYS. Following IVM, oocytes were assessed for maturation.

UnSt-GV oocytes and a proportion of UnSt-MII oocytes were processed for GSH assay. A proportion of UnSt-MII oocytes from the COC groups were inseminated and fertilisation outcomes recorded as described above.

Experiment 5: maturation and GSH content of immature oocytes derived from stimulated macaque ovaries matured in the presence or absence of CYS

Immature oocytes (St-GV) with intact cumulus vestment were collected from gonadotrophin-stimulated ovaries. Oocytes were matured in mCMRL-1066 (seven replicates) in the presence or absence of 100 μ M CYS for 30 h. Following IVM, oocytes were denuded of cumulus cells and assessed for maturation. St-GV-MII oocytes were processed for GSH assay as described above (four to five replicates).

Statistical analysis

Differences in maturation and fertilisation outcomes in each treatment group, expressed as a proportion of total oocytes, were compared using Chi-squared or Fisher's exact tests. Oocyte GSH content was compared using ANOVA and the post hoc Tukey test or by *t*-test. GSH outliers were excluded by applying Chauvenet's criteria ($\text{mean} \pm 1.645 \times \text{s.d.}$) before comparison by ANOVA. Non-proportional data are expressed as the mean $\pm$ s.e.m. Significance was set at $P < 0.05$.

Results

Because no significant differences were observed in the UnSt-MII maturation rate, UnSt-MII fertilisation outcome or UnSt-GV and UnSt-MII GSH content between *M. nemestrina* and *M. fascicularis*, data for the two species were pooled in Experiments 1, 2 and 4.

Experiment 1: effect of time on maturation and fertilisation of macaque oocytes from unstimulated ovaries

The UnSt-MII rate was significantly lower at 24 h compared with 30 h (Table 1). There was no significant difference in the number of activated oocytes (2PN and 1PN) between the 24 and 30 h IVM treatment groups (78.9% and 79.0%, respectively). However, at both time points, 2PN formation was significantly lower than 1PN formation (Table 1).

In total, 26 1PN and 17 FF oocytes were assessed by immunofluorescence. Twenty of the 26 1PN oocytes (76.9%) had a penetrated spermatozoon that was either completely condensed or in a state of partial decondensation (Fig. 1a). The remaining six 1PN oocytes (23.1%) failed to exhibit evidence of sperm penetration and were classified as SpA. The sperm head was always in close association with the female PN and on the same focal plane. None of the experimental oocytes had retained female chromatin in a condensed state (MII). Of the 17 FF oocytes analysed, 16 (94.1%) were still at the MII stage (Fig. 1b) and one (5.9%) had undergone activation due to sperm penetration but had failed to form pronuclei.

Experiment 2: comparison of media for maturation and fertilisation of macaque oocytes from unstimulated ovaries

Maturation rates and fertilisation outcomes for UnSt-GV oocytes cultured in mHECM-10 and mCMRL-1066 are given in Table 2. Briefly, the UnSt-MII rate was significantly greater for oocytes cultured in mCMRL-1066 medium compared with mHECM-10 medium. There was

a significant increase in the rate of 2PN formation in UnSt-MII oocytes matured in mCMRL-1066 compared with UnSt-MII oocytes matured in mHECM-10.

Experiment 3: comparison of fertilisation outcome and GSH content of IVO and IVM macaque oocytes

Following ovarian stimulation, 461 oocytes were retrieved from 16 *M. nemestrina* females (averaging 28.8 ± 5.0 per cycle), whereas 815 oocytes were retrieved from 11 *M. fascicularis* females (averaging 74.1 ± 8.7 per cycle). The stimulation protocol ensured that there were significantly more St-MII oocytes at the time of collection (64.6% to 65.9% for *M. nemestrina* and *M. fascicularis*, respectively) than St-GV (15.4% to 16.1% for *M. nemestrina* and *M. fascicularis*, respectively) or St-MI (18.0% to 20.0% for *M. nemestrina* and *M. fascicularis*, respectively) oocytes, with no significant difference in the St-MII rate between species (Table 3). The UnSt-MII rates for *M. nemestrina* and *M. fascicularis* were equivalent. The UnSt-MII rate was significantly lower compared with St-MII-derived oocytes for both species (Table 3).

The fertilisation outcome between St-MII and UnSt-MII oocytes for each species is given in Table 3. Both *M. nemestrina* and *M. fascicularis* produced significantly more St-MII zygotes displaying 2PN formation compared with 1PN, FF or polyspermy. The 2PN rate did not differ significantly between species (Table 3). Both *M. nemestrina* and *M. fascicularis* UnSt-MII zygotes had a significantly greater proportion of 1PN and a significantly lower proportion of 2PN zygotes following insemination compared with respective St-MII oocytes (Table 3). The FF rate was significantly higher for UnSt-MII oocytes compared with St-MII oocytes (Table 3).

Because of the limited number of *M. nemestrina* St-MII oocytes (~18 oocytes per female) only *M. fascicularis* St-MII oocytes were available for GSH assay. There was no significant difference in GSH content between St-GV and UnSt-GV *M. fascicularis* oocytes (4.05 ± 0.19 v. 4.20 ± 0.13 pmol per oocyte, respectively; $n = 20-60$). The GSH content of *M. fascicularis* St-MII oocytes was significantly higher than that of *M. fascicularis* UnSt-MII oocytes (7.52 ± 0.34 v. 4.75 ± 0.35 pmol per oocyte, respectively; $n = 18-36$). The GSH content of IVO oocytes increased significantly between the GV and MII stages (from 4.05 ± 0.19 to 7.52 ± 0.34 pmol per oocyte, respectively; $n = 36-60$). There was no significant difference in the GSH content between *M. nemestrina* and *M. fascicularis* UnSt-GV oocytes (4.33 ± 0.13 v. 4.20 ± 0.13 pmol per oocyte, respectively; $n = 20-27$) or UnSt-MII oocytes (3.82 ± 0.74 v. 4.75 ± 0.35 pmol per oocyte, respectively; $n = 16-18$). Furthermore, the GSH content of the UnSt-GV oocytes was not significantly different to the GSH content of the UnSt-MII oocytes for either species.

Experiment 4: maturation, GSH content and fertilisation outcome of COCs and DO matured in the presence or absence of CYS

In the present study, CYS had no effect on the UnSt-MII rate of either the DO or COC treatment groups (Table 4). Significantly more COCs progressed to the MII stage after 30 h maturation compared with DOs (Table 4).

Supplementation with CYS during COC culture significantly increased the GSH content of UnSt-MII oocytes compared with medium lacking CYS (Table 4). The GSH content of control and CYS-supplemented DOs was significantly lower at the MII stage compared with the GV stage (Table 4). Supplementation with CYS did not significantly increase the GSH content of DO UnSt-MII oocytes compared with control DOs (Table 4).

Maturation of COCs in the presence or absence of CYS had no significant effect on the rate of either 2PN formation (14/44 (31.8%) v. 13/51 (25.5%), respectively) or FF (23/44 (52.3%) v. 22/51 (43.1%), respectively). A reduction in the rate of 1PN formation in the COC + CYS group compared with control COC approached significance (7/44 (15.9%) v. 16/51 (31.4%), respectively; $P = 0.056$).

Experiment 5: maturation and GSH content of immature oocytes derived from stimulated macaque ovaries matured in the presence or absence of CYS

The maturation rate of St-GV oocytes was not significantly different to the maturation rate of *M. nemestrina* and *M. fascicularis* UnSt-GV (Table 3) oocytes (19/42 (45.2%) v. 109/193 (56.5%) and 71/132 (53.8%), respectively; $\chi^2 = 1.771$, d.f. = 2, $P = 0.4125$). CYS supplementation did not significantly affect the St-GV-MII rate compared with control (19/42 (45.2%) v. 19/43 (44.2%), respectively). There was no significant difference in the GSH content of St-GV-MII oocytes following maturation in the presence ($n = 12$) or absence ($n = 7$) of CYS (8.04 ± 1.54 v. 7.33 ± 0.38 pmol per oocyte, respectively). The GSH content of St-GV-MII oocytes matured in mCMRL-1066 medium was equivalent to that of St-MII oocytes (7.52 ± 0.34 pmol per oocyte; Table 4).

Discussion

Although pregnancies and live births have been obtained from IVM oocytes collected from unstimulated cycles in the human (Smith *et al.* 2000; Chian *et al.* 2004; Söderström-Anttila *et al.* 2005), to date the only macaque infants born from IVM oocytes were produced following ovarian priming with gonadotrophins (Schramm and Paprocki 2000). The inadequacies of the IVM systems in both the human and NHP are evidenced by fertilisation failure and poor embryonic development (Morgan *et al.* 1991; Zheng *et al.* 2001; Schramm *et al.* 2003), suggesting that cytoplasmic maturation is incomplete.

In order to improve the developmental potential of the IVM NHP oocyte, an optimised maturation system is necessary. Maturation studies in the NHP state a range of culture times (24–50 h), with no clear consensus on optimal timing for oocyte IVM (Morgan *et al.* 1991; Hewitson *et al.* 1996; Zheng *et al.* 2001; Delimitreva *et al.* 2006). In *M. mulatta*, the mean interval for first polar body extrusion in oocytes derived from unstimulated ovaries is 34 h (Schramm *et al.* 1994). In the present study, based on our previous observations in *M. nemestrina* and *M. fascicularis*, we compared maturation times of 24 and 30 h. Almost one-fifth of oocytes had completed nuclear maturation by 24 h, with maximal UnSt-MII rates observed at 30 h, a slightly shorter time compared with *M. mulatta*. Fertilisation outcomes were equivalent between these two time points, suggesting that the increased culture time was not detrimental to oocyte quality in relation to sperm penetration and pronuclear formation. Further culture of immature oocytes identified at 30 h as either GV or MI until 36 h did not significantly increase the proportion of UnSt-MII oocytes (data not shown). Because these results indicated high rates of nuclear maturation following 30 h culture in the presence of cumulus cells and hormones, this protocol was used for subsequent IVM experiments. However, fertilisation outcomes were suboptimal and further modifications to the maturation system were investigated.

Culture media can have differing effects on oocyte maturation and embryo development (Yoshida *et al.* 1992; Zheng *et al.* 2001; Roberts *et al.* 2002; Schramm *et al.* 2003). Both HECM-10 and CMRL-1066 media have been used previously for the IVM of *M. mulatta* oocytes (Morgan *et al.* 1991; Schramm and Bavister 1995; Zheng *et al.* 2001). Although previous comparison of *M. mulatta* oocyte maturation in a simple (TALP) v. complex (CMRL-1066) medium suggested no differences in oocyte quality, a direct comparison of normal and abnormal fertilisation rates was not made (Morgan *et al.* 1991). In the present study,

maturation rate and fertilisation were compared for oocytes subjected to IVM in mCMRL-1066, a complex medium containing necessary substrates for GSH synthesis, with those matured in mHECM-10, which lacks cysteine. The maturation and normal fertilisation rates, as evidenced by two pronuclei, were higher for UnSt-MII oocytes matured in mCMRL-1066 medium compared with mHECM-10. Although oocyte GSH content was not compared between these two media, the absence of cysteine possibly restricted GSH synthesis and reduced synchronous pronuclear formation for oocytes matured in mHECM-10. However, the ability of the UnSt-MII oocyte matured in mCMRL-1066 to undergo synchronous pronuclear formation was also reduced compared with the St-MII oocyte.

Immunofluorescence of UnSt-MII zygotes displaying only 1PN following insemination revealed a high proportion of interphase failure (77%) where the sperm head remained condensed in the presence of the female pronucleus. In *M. mulatta* IVM oocytes, 58% of all oocytes that exhibit 1PN formation following intracytoplasmic sperm injection (ICSI) are the result of failed MPN formation (Hewitson *et al.* 1996). Because the incidence of interphase failure following both IVF and ICSI of macaque IVM oocytes is similar, the failure of MPN formation is likely the result of IVM rather than the method of insemination.

Failure of MPN formation is an error associated with incomplete cytoplasmic maturation, and the depletion of oocyte GSH may be one of the contributing factors to failure of MPN formation in the macaque IVM oocyte. In the pig, oocyte GSH content is correlated with subsequent fertilisation and developmental success (Yamauchi and Nagai 1999), with significant differences evident between IVO and IVM oocytes (Brad *et al.* 2003). In the present study, the fertilisation outcome and GSH levels of St-MII and UnSt-MII oocytes were compared in order to determine whether the different fertilising capacity of the MII oocytes could be explained by variations in GSH content. Although both IVO- and IVM-derived oocytes had equivalent GSH content at the GV stage, the IVM oocytes had significantly less GSH at the MII stage. Environmental factors associated with *in vitro* culture, such as increased oxidative stress (Van Soom *et al.* 2002), the breakdown of cumulus–oocyte gap junctions (Mattioli *et al.* 1988; Maedomari *et al.* 2007) or insufficient exogenous GSH substrates, may be responsible for this loss in GSH content during the final stage of maturation.

In several species, the addition of CYS to the IVM medium enhances the GSH content of the mature oocyte (de Matos *et al.* 1997, 2002; Bing *et al.* 2002), with subsequent improvement in fertilisation and embryo development (Gruppen *et al.* 1995; Bing *et al.* 2002; de Matos *et al.* 2002; Luciano *et al.* 2005). Effective concentrations of CYS range from 50 to 200 μ M and, for comparison, 100 μ M CYS was selected for use in the present study. The addition of CYS increased GSH levels in UnSt-MII oocytes compared with control. However, GSH levels were still significantly lower than in St-MII oocytes and, as such, only a slight improvement in fertilisation, as evidenced by a lower incidence of 1PN formation compared with control UnSt-MII zygotes, was observed. Because normal fertilisation rates were not significantly improved, full cytoplasmic maturation of the cumulus-enclosed macaque IVM oocyte may not have been adequately supported by the selected concentration of CYS.

Immature oocytes retrieved during stimulated ovarian cycles and matured in mCMRL-1066 medium (St-GV-MII) demonstrated equivalent rates of maturation and higher MII oocyte GSH content compared with UnSt-MII oocytes. Because the GSH content of GV oocytes from stimulated and unstimulated ovaries is similar, the discrepancy between MII oocyte GSH levels following IVM suggests that there may be an association between GSH synthesis and gonadotrophin stimulation. In the rat, expression of ovarian GCL and follicular GSH levels change throughout the oestrous cycle and are significantly increased during pregnant mare's serum gonadotropin-stimulated follicular growth (Luderer *et al.* 2001; Tsai-Turton and Luderer

2005). Because unstimulated ovaries were collected from females of unknown cycle stage, it is not clear whether GSH levels in UnSt-MII oocyte were influenced by the oestrous cycle.

Cumulus cells play an important role in GSH synthesis, promoting the uptake of cysteine by oocytes through gap junction communication (Mori *et al.* 2000). Conversely, DOs have a limited ability to synthesise GSH and exhibit reduced developmental capability (de Matos *et al.* 1997; Curnow *et al.* 2010). Studies in the cow and goat have shown increased GSH content and embryo development when DOs are matured in the presence of CYS (Luciano *et al.* 2005; Zhou *et al.* 2008). In the present study, the maturation rate of DOs was significantly lower than that of COCs and GSH levels were significantly lower at the MII stage compared with the immature oocyte at collection, suggesting that GSH synthesis in the DO is perturbed.

The γ -glutamyl cycle is responsible for the maintenance of oocyte GSH, is energy dependent and requires sufficient ATP for GSH synthesis to occur (Deneke and Fanburg 1989). In the absence of cumulus cells, energy metabolism of the DO is disrupted (Downs 1995), possibly limiting GSH synthesis even in the presence of sufficient substrate. It is unclear from the present results what the rate-limiting factors for GSH synthesis in the DO were; however, it would appear that species differences in CYS utilisation may exist.

There are conflicting results in the literature with respect to the efficacy of CYS as both a modulator of oocyte GSH content and promoter of embryo development. In the bovine and ovine, CYS addition to IVM medium results in higher GSH content and improved blastocyst development in both COC and DO (de Matos *et al.* 1996, 2002; Luciano *et al.* 2005). In the goat, CYS supplementation improves both MPN formation and embryo development of the IVM oocyte, despite non-significant changes in oocyte GSH content (Rodríguez-González *et al.* 2003). Although the addition of cysteine (0.04–3.3 mM) alone increases GSH content of IVM pig oocytes (Yoshida 1993; Rodríguez-González *et al.* 2003; Whitaker and Knight 2004), there is no improvement in pig oocyte GSH when CYS (150 μ M) is used in combination with high levels (3.3 mM) of cysteine (Whitaker and Knight 2004). Because CMRL-1066 contains a comparable level of cystine and intermediate levels of cysteine (0.11 and 1.48 mM, respectively) compared with media used in other studies (e.g. 0.11 and 0.57 μ M, respectively, in TCM-199), it is possible that the interaction of CYS and cysteine in mCMRL-1066 minimised the improvement in oocyte GSH content and subsequent fertilisation outcomes.

The results presented in this study emphasise the discrepancy in GSH content between IVO and IVM macaque oocytes and suggest that GSH synthesis is a key component for improvement of IVM outcomes. Because the NHP is considered a useful model for the study of human oocyte and embryo development, further research into the manipulation of the γ -glutamyl cycle of NHP oocytes during IVM may have important implications within both the research and clinical settings.

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Fig. 1.

(a) Fluorescent microscope image of macaque IVM oocyte displaying one pronuclei after fertilisation. Green, tubulin; blue, chromatin. FPN, female pronucleus; S, partially decondensed sperm head; PB, polar body. Bar = 25 μm . (b) Fluorescent microscope image of a macaque IVM oocyte assessed as failed to fertilise 12–15h after insemination. MII, chromatin in MII (blue) aligned on spindle (tubulin; green); PB, polar body. Bar = 50 μm .

Table 1

Maturation and fertilisation outcomes of macaque oocytes derived from unstimulated ovaries and assessed after 24 or 30 h of maturation

Data show the number of oocytes in each group, with percentages given in parentheses. Within rows, values with different superscript letters differ significantly ($P < 0.05$); within columns, values with different symbols differ significantly ($P < 0.05$). 2PN, two pronuclei and at least one polar body; 1PN, one pronucleus and at least one polar body; FF, failed fertilization

Maturation	Maturation rate		Fertilisation outcome		
	MII	2PN	1PN	FF	Total Oocytes
24h	19/96* (19.8)	1 ^a (5.3)	14 ^b (73.7)	4 ^a (21.1)	19
30h	62/96 [†] (64.6)	6 ^a (9.7)	43 ^b (69.4)	13 ^a (21.0)	62

Table 2
Comparison of maturation rate and fertilisation outcomes of macaque oocytes derived from unstimulated ovaries matured in mHECM-10 or mCMRL-1066 medium for 30 h

Unless indicated otherwise, data show the number of oocytes in each group, with percentages given in parentheses. Within rows and columns, values with different superscript letters differ significantly ($P < 0.05$). GVBD, germinal vesicle breakdown; 2PN, two pronuclei and at least one polar body; 1PN, one pronucleus and at least one polar body

Medium	Maturation rate			Fertilisation outcome		
	GVBD	MI	Total no. oocytes	2PN	1PN	Total no. oocytes
mHECM-10	234 ^a (62.7)	170 ^b (45.6)	373	13 ^a (7.8)	64 ^b (38.6)	166
mCMRL-1066	146 ^a (70.5)	118 ^c (57.0)	207	25 ^b (24.3)	31 ^b (30.1)	103

Table 3

Comparison of maturation rate and fertilisation outcome of *Macaca nemestrina* and *Macaca fascicularis* oocytes matured *in vivo* or *in vitro*

Unless indicated otherwise, data show the number of oocytes in each group, with percentages given in parentheses. Within rows and columns, values for each outcome measure for each species with different superscript letters differ significantly ($P < 0.05$). 2PN, two pronuclei and two polar bodies; IPN, one pronucleus and two polar bodies; FF, failed fertilisation; IVO, *in vivo*-matured oocytes ($n = 11-16$ replicates); IVM, *in vitro*-matured oocytes ($n = 3-9$ replicates). IVM oocytes were matured in mCMRL-1066 + fetal calf serum for 30 h

	Maturation rate (%)		Fertilisation outcome				Total no. oocytes
	MI	2PN	1PN	>2PN	FF		
<i>Macaca nemestrina</i>							
IVO	298/461 ^a (64.6)	208 ^a (72.0)	12 ^c (4.2)	30 ^{bc} (10.4)	39 ^c (13.5)	289	
IVM	109/193 ^b (56.5)	26 ^b (30.2)	26 ^b (30.2)	0 ^a (0.0)	34 ^b (39.5)	86	
<i>Macaca fascicularis</i>							
IVO	537/815 ^a (65.9)	357 ^a (71.4)	9 ^c (1.8)	26 ^d (5.2)	108 ^b (21.6)	500	
IVM	71/132 ^b (53.8)	9 ^b (22.0)	10 ^b (24.4)	2 ^d (4.9)	20 ^c (48.8)	41	

Table 4
Maturation rate and glutathione content of macaque cumulus-enclosed or -denuded oocytes
matured in the presence or absence of 100 μ M cysteamine

Unless indicated otherwise, data show the number of oocytes in each group, with percentages given in parentheses ($n = 5$ –20 replicates). Within rows and columns, values with different superscript letters differ significantly ($P < 0.05$). COC, cumulus–oocyte complexes; DO, denuded oocytes; GSH, glutathione; CYS, cysteamine; GV, immature oocytes at the time of collection from unstimulated ovaries; MII, mature oocytes assayed after 30 h maturation in mCMRL-1066 + fetal calf serum

	Maturation rate	GSH content (pmol per oocyte)	
	MI	GV	MI
COC	115/284 ^a (40.5)	4.22 $\pm$ 0.10 ^a (81)	3.59 $\pm$ 0.10 ^a (46)
COC + CYS	140/332 ^a (42.2)	4.54 $\pm$ 0.10 ^{ab} (119)	4.96 $\pm$ 0.35 ^b (70)
DO	35/141 ^b (24.8)	4.02 $\pm$ 0.28 ^a (112)	2.63 $\pm$ 0.66 ^c (29)
DO + CYS	26/136 ^b (19.1)	4.02 $\pm$ 0.28 ^a (112)	2.53 $\pm$ 0.62 ^c (23)