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Primate model of MI oocyte *in vitro* maturation and the effects of a novel glutathione donor on maturation, fertilization and blastocyst development

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Abstract

Objective—To assess the effect of glutathione ethyl ester (GSH-OEt) on the development of macaque MI oocytes as a model for human MI oocyte *in vitro* maturation.

Design—Prospective cohort study

Setting—Non-human primate ART program.

Animal(s)—23 *Macaca fascicularis* females aged 6.5–12.5 years

Intervention(s)—Ovarian stimulation and maturation of MI oocytes in i) HTF, ii) mCMRL-1066, iii) mCMRL-1066+GSH-OEt 3mM or iv) mCMRL-1066+GSH-OEt 5mM. Oocytes were assessed for maturation after 4–6 h (early) and 18–20 h (late) of culture. Mature oocytes were inseminated or subjected to glutathione (GSH) assay. Zygotes were cultured to the blastocyst stage for total differential cell counts.

Main Outcome Measure(s)—Oocyte maturation rate, GSH content, pronuclear formation and blastocyst development and cell number were compared between IVM treatment groups and sibling *in vivo* matured (IVO) MII oocytes.

Result(s)—Compared to HTF, mCMRL-1066 supported higher rates of normal fertilization and blastocyst development in early but not late maturing MI-MII oocytes. 5 mM GSH-OEt significantly increased blastocyst total cell and ICM cell number in early MI-MII oocytes compared to IVO and IVM controls. GSH-OEt significantly increased oocyte GSH content and fertilization in late maturing oocytes but not blastocyst development.

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Declaration of interest The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Conclusion(s)—GSH-OEt positively effects development of early and late maturing IVM oocytes.

Keywords

Glutathione ethyl ester; non-human primate; oocyte in vitro maturation; male pronucleus formation

INTRODUCTION

Ovarian stimulation cycles in both the human and non-human primate (NHP) yield mature and immature oocytes (1,2,3). To maximize embryo number from each cycle the potential of IVM MI oocytes to contribute to the cohort of available embryos is of interest within the human clinical and NHP ART research settings (4,5). However, the vast majority of research in the human indicates that IVM MI oocytes exhibit reduced fertilization (4,6), embryo quality (7,8) and pregnancy outcomes (9).

The immature oocyte requires co-ordination of both nuclear and cytoplasmic maturation for normal fertilization and embryo development. Intracellular glutathione (GSH) content is a potential marker of oocyte cytoplasmic maturation and developmental competence (10). During oocyte maturation the γ -glutamyl cycle is responsible for *de novo* synthesis of GSH which is responsible for protection against oxidative stress (11,12), male pronucleus formation (13,14,15) and embryo development to the blastocyst stage (16,17,18,19).

The *in vitro* culture environment during oocyte maturation plays a crucial role in promoting or depleting oocyte GSH content. The composition of IVM medium and the presence or absence of cumulus cells greatly influences oocyte GSH synthesis (18,20,21,22). Removal of cumulus cells for evaluation of oocyte nuclear status during ICSI cycles limits the capability of the oocyte to synthesize GSH (23). Incubation of denuded human oocytes in IVF medium results in a depletion of GSH content as a result of reactive oxygen species (ROS) generation and, the extent of which is dependent on media formulation (24).

Traditional approaches to cellular GSH loading rely on the γ -glutamyl cycle and are limited by the regulation of GSH synthesis through feedback inhibition (25). In a previous study (26) we identified a cell permeable GSH donor capable of increasing the GSH content of both cumulus-enclosed and cumulus-free IVM bovine oocytes. In this study the NHP was used as a model of MI oocyte IVM in an experimental setting. We examined the role of media formulation and the addition of a GSH donor, GSH-OEt, on the relationship between oocyte GSH content and IVF outcome of *Macaca fascicularis* cumulus free MI oocytes. Oocyte maturation, GSH content, fertilization, and blastocyst development was compared to sibling IVO oocytes as a model for the improvement of clinical MI oocyte IVM.

MATERIALS AND METHODS

Except where otherwise indicated chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All animal studies were approved by the University of Washington Institutional Animal Care and Use Committee (3387-01).

Ovarian stimulation and oocyte retrieval

Adult female *M. fascicularis* (n=23) with regular menstrual cycles, aged 6.5 to 12.5 yrs and weighing from 2.5 to 4.9 kg, were housed at the Washington National Primate Research Center (WaNPRC).

Oocyte donors were subjected to ovarian stimulation as described previously (27). Oocyte recovery, sperm collection, insemination, and embryo culture have been described previously (27). Follicular aspirates were collected into Hepes modified Human Tubal Fluid (Irvine Scientific, Santa Ana, CA, USA) supplemented with 0.3% w/v bovine serum albumin (mHTF+BSA). Oocytes were treated with hyaluronidase (40 iu/ml) to remove cumulus cells and classified according to nuclear maturation status (germinal vesicle, GV; Metaphase I, MI; Metaphase II, MII). Metaphase II oocytes were transferred to 20 μ l drops of fertilization media (Human Tubal Fluid supplemented with 0.3% w/v BSA; HTF+BSA) under mineral oil and incubated in a humidified atmosphere of 6% CO₂ in air at 37°C for 1-2 h prior to insemination.

Oocyte in vitro maturation

Cumulus free MI oocytes were randomly assigned to maturation in either HTF+BSA or Connaught Medical Research Laboratories medium (Invitrogen, Carlsbad, CA, USA) supplemented with L-glutamine (0.4 mM), pyruvate (0.25 mM), gentamycin sulfate (50 μ g/ml) and 0.4 % w/v BSA (mCMRL-1066). Concentrations of 3 mM and 5 mM GSH-OEt were prepared in mCMRL-1066. Oocytes were cultured in maturation medium (400 μ l) overlaid with mineral oil in individual wells (NUNC 4-well dish; Thermo Fischer Scientific, Rochester, NY, USA) in a humidified atmosphere of 6% CO₂ in air at 37°C. At 4-6 h of culture oocytes were assessed for polar body (PB) extrusion (early maturing MI-MII). MII oocytes were removed and transferred to HTF+BSA for insemination while remaining MI oocytes were reassessed following overnight culture at 18-20 h post maturation (late maturing MI-MII). MII oocytes were either prepared for GSH assay or inseminated.

Intracytoplasmic sperm injection

Frozen/thawed epididymal spermatozoa were prepared by centrifugation (500 g) on a single 80% Puresperm® density gradient (NidaCom laboratories, Gothenburg, Sweden) for 10 min at RT. The sperm were retrieved and re-pelleted by centrifugation at 400 g for 5 min in mHTF+BSA. Final sperm preparations were held in mHTF+BSA at RT for 30 min prior to 1:4 dilution with 10% (w/v) polyvinylpyrrolidone (PVP; Irvine Scientific, Santa Ana, CA) and used for ICSI.

Following ICSI, injected oocytes were transferred to individual 20 μ l drops of HTF+BSA under mineral oil in a humidified atmosphere of 6% CO₂ in air at 37°C. Fertilization was assessed visually at 12-15 h post-injection by the presence of pronuclei (PN) and PBs and recorded as 2PN; 1PN; >2PN (polyspermy) or FF (failed fertilization; no pronuclei, 1 PB).

In vitro embryo culture

Zygotes displaying 2PN after insemination (Day 1) were grouped (3 to 5 embryos / 50 μ l drop) and cultured in Hamster Embryo Culture Medium-9 supplemented with 0.4% w/v BSA (HECM-9+BSA) and 0.5 mM glucose under mineral oil in a humidified atmosphere of 6% CO₂, 7% O₂, 87% N₂ at 37°C. On Day 3 embryos were assessed for cleavage and transferred to HECM-9+BSA supplemented with 3 mM glucose and cultured within individual microwells (diameter; 200 μ m, depth; 260 μ m) of a polydimethylsiloxane membrane (PDMS) insert in a 4-well dish (28). Media was changed every 48 h until Day 9 of culture when embryos were assessed for blastocyst development.

GSH sample preparation and analysis

Preparation of oocytes for GSH analysis has been described previously (27). The total GSH content of oocytes (1-6 oocytes / tube) was determined using a commercial 5,5'-dithio-bis(2-

nitrobenzoic acid) (DTNB)-GSH reductase recycling assay kit (Northwest Life Science, Vancouver, WA, USA). Samples and standards were analysed at 405 nm with repeated reads at 2 min intervals for 30 min and the rate of change of absorbance was determined by linear regression analysis for each sample and blank. Concentrations of total GSH (pmol/oocyte) were calculated from the standard curve. Inter-assay CV was 9.1% across 12 replicate plates.

Differential Staining of Blastocysts

Day 9 blastocysts from control IVO, mCMRL-1066 control and GSH-OEt treatment groups were subject to differential cell staining using propidium iodide (PI; 100 µg/ml) and bisbenzimidazole (Hoechst 33258; 25 µg/ml) according to the methods described by Thouas *et al* (29). Prepared blastocysts were mounted in a drop of 100% glycerol and total cell number (TCN; Hoechst) and trophectoderm cell counts (TE; PI) were performed using an inverted microscope with UV lamp and appropriate filters.

Statistical Analysis

Maturation, fertilization and blastocyst development in each treatment group were expressed as a proportion of total oocytes and differences were compared using Chi-square or Fisher Exact test. For blastocyst development data only MI-MII oocytes with blastocyst competent sibling MII oocytes were included in the data analysis. Oocyte GSH content and blastocyst cell number were analyzed using t-test or ANOVA and post-hoc Tukey test as appropriate. GSH outliers were excluded by applying Chauvenet's criteria ($\text{mean} \pm 1.645 \times \text{Standard Deviation}$) before comparison by ANOVA. Non-proportional data are expressed as mean $\pm$ s.e.m. Significance of differences were determined at $p < 0.05$.

RESULTS

A total of 2148 oocytes were retrieved from 23 ovarian stimulations (93.4 ± 8.0 oocytes / female; range; 29-176). Of these 870 oocytes were at the MII-stage ($39.4 \pm 4.2\%$) and 693 at the MI-stage ($34.6 \pm 4.3\%$). 617 of the 693 MI oocytes (89.0%) were randomly allocated to IVM treatment groups. Less than 2.5% of oocytes (49/2148) were classified as degenerate or not fully grown and were excluded.

Maturation, GSH content, fertilization and development of early maturing (4-6 hr) MI-MII oocytes

Oocytes cultured in mCMRL-1066 had a significantly lower rate of maturation compared to oocytes cultured in HTF (Table 1). The addition of GSH-OEt to mCMRL-1066 at 3 mM and 5 mM restored the MI-MII rate to levels observed in the HTF group (Table 1).

There was no significant difference in oocyte GSH content between oocytes matured in HTF, mCMRL-1066 or mCMRL-1066 supplemented with 3 mM or 5 mM GSH-OEt (5.00 ± 0.68 to 6.75 ± 0.56 pmol/oocyte, $n=9-13/\text{group}$) and early maturing MI-MII GSH data was pooled for further analysis. There was no significant difference in the GSH content of early maturing MI-MII oocytes compared to IVO MI oocytes at collection (5.93 ± 0.37 v. 5.78 ± 0.21 pmol/oocyte, $n=35-43$). Early maturing MI-MII oocytes had significantly higher and lower GSH content, respectively compared to IVO GV and MII oocytes (5.93 ± 0.37 v. 4.43 ± 0.10 and 7.28 ± 0.39 $n=32-44$).

In both the IVO and IVM groups the 2PN rate was significantly higher than the incidence of abnormal (1PN, >2PN) and failed fertilization (FF) (Table 1). Oocytes matured in HTF had similar 2PN rates, but significantly higher 1PN rates compared to IVO oocytes (Table 1). Oocytes matured in HTF had significantly lower 2PN and higher 1PN rates compared to

oocytes matured in mCMRL-1066 in the absence or presence of GSH-OEt (Table 1). Oocytes matured in mCMRL-1066 had a significantly higher 2PN rate compared to IVO oocytes (Table 1). Maturation of oocytes in control mCMRL-1066 medium significantly reduced the incidence of failed fertilization compared to IVO oocytes (Table 1).

The blastocyst rate for oocytes matured in HTF was significantly lower compared to IVO oocytes (Table 2). There was no significant difference in the blastocyst rate for oocytes IVM in mCMRL-1066 in the presence or absence of GSH-OEt (Table 2) which were equivalent to the blastocyst rates of IVO oocytes and significantly higher than oocytes IVM in HTF (Table 2).

Differential staining of blastocysts revealed a significant increase in both TCN and inner cell mass (ICM) cell number for blastocysts derived from oocytes IVM in 5 mM GSH-OEt compared to control mCMRL-1066 IVM and IVO oocytes (Table 2). There was a significant linear trend for an increase in TCN of IVM oocytes cultured in mCMRL-1066 with increasing concentration of GSH-OEt ($F=5.22$, $p=0.04$).

Maturation, GSH content, fertilization and development of late maturing (18-20 hr) MI-MII oocytes

There was no significant difference in the MI-MII rate between HTF and mCMRL-1066 treatment groups. Oocytes matured in the presence of 3 mM and 5 mM GSH-OEt had significantly higher rates of MI-MII maturation compared to mCMRL-1066 control (Table 3).

The GSH content of IVO derived GV, MI and MII oocytes at the time of oocyte retrieval significantly increased with stage of maturation reaching peak GSH content at MII (Figure 1). The GSH content of late maturing MI-MII oocytes from all IVM treatment groups were significantly lower than the GSH content of IVO MI and MII oocytes (Figure 1). Late maturing MI-MII oocytes cultured in HTF had a significantly lower GSH content compared to all other IVM treatment groups (Figure 1). The addition of 5 mM GSH-OEt significantly increased the GSH content of the late maturing MI-MII oocyte compared to all other treatment groups (Figure 1).

Late maturing MI-MII oocytes cultured in HTF and mCMRL-1066 had lower 2PN and higher 1PN fertilization rates compared to IVO oocytes (Table 3). The 2PN and 1PN fertilization rates for late maturing MI-MII oocytes cultured in the presence of 3 mM and 5 mM GSH-OEt were equivalent to IVO oocytes (Table 3). While both 3 mM and 5 mM GSH-OEt significantly reduced the incidence of 1PN formation, 5 mM GSH-OEt also significantly increased the 2PN rate compared to control mCMRL-1066 MI-MII oocytes (Table 3).

There was no significant difference in the rate of blastocyst development between late maturing MI-MII treatment groups (Table 3). The blastocyst rate for oocytes IVM with 3 mM GSH-OEt was not significantly different to IVO oocytes (Table 3).

Analysis of blastocyst differential cell counts for late maturing MI-MII oocytes cultured in mCMRL-1066 with or without GSH-OEt supplementation indicated no significant difference between IVO and IVM treatment groups for the TCN (125.17 ± 14.93 to 142.50 ± 20.36 cells, $n=3-6/\text{group}$), the number of ICM cells (38.69 ± 4.92 to 58.17 ± 10.76 cells, $n=3-6/\text{group}$), the number of TE cells (84.33 ± 20.46 to 107.97 ± 12.21 cells, $n=3-6/\text{group}$) or the ICM:TE ratio (1.53 ± 0.47 to 2.95 ± 0.29 , $n=3-6/\text{group}$).

DISCUSSION

When few or no MII oocytes are observed following oocyte retrieval in human IVF cycles, MI oocytes derived from those cycles, and matured *in vitro*, represent a potential source of viable oocytes for embryo production (4,7,9,30,31). In this study the NHP was used as a model of gonadotrophin primed MI oocyte IVM in an experimental setting by comparing simple v. complex media formulation and exploring the relationship between oocyte GSH content and IVF outcome.

Although HTF supported higher rates of early MI-MII maturation, subsequent fertilization and blastocyst development was significantly impaired compared with oocytes cultured in mCMRL-1066. The improved outcomes of mCMRL-1066 early maturing MI-MII oocytes were not associated with increased oocyte GSH content suggesting that other components of mCMRL-1066, such as full amino acid complement, may have been responsible for supporting oocyte cytoplasmic maturation and, improved pronuclear formation and blastocyst development (32).

The GSH content of early maturing MI-MII oocytes was not significantly different between IVM treatment groups and was comparable to levels observed in IVO MI oocytes suggesting that early MI-MII macaque oocytes are able to maintain their GSH reserves, perhaps through GSH/GSSG recycling or through sufficient levels of intracytoplasmic cysteine that support short term GSH synthesis (33). However, the addition of GSH-OEt did not significantly elevate GSH content of early MI-MII oocytes. While the majority of GSH-OEt is taken up rapidly and deesterified within a matter of hours (34) a proportion of GSH-OEt remains intact and intracellular levels of cysteine derived from GSH metabolism increase (35) without raising GSH levels, suggesting a possible indirect effect of GSH-OEt on cellular redox status.

GSH-OEt significantly improved the maturation rate of both early and late maturing MI-MII oocytes compared to mCMRL alone but not HTF thus indicating that GSH-OEt was able to overcome the maturation inhibiting nature of mCMRL-1066. The mechanism by which GSH-OEt exerted its positive influence on early MI-MII maturation rates in the absence of an increase in total GSH content is unclear, however the positive effect of GSH-OEt extended to an increase in blastocyst cell count similar to the observed effect of GSH-OEt on blastocyst cell counts in IVM bovine oocytes (36).

Although the GSH-OEt mediated improvement in oocyte maturation, fertilization and blastocyst cell counts were not directly related to the measure of total oocyte GSH content, they may relate to an alteration of the intracellular ratio of reduced to oxidized GSH (GSH:GSSG). In mouse oocytes with equivalent total GSH content, a shift towards higher GSH:GSSG ratio in *in vitro* aged oocytes was associated with significantly higher fertilization and blastocyst development compared to *in vivo* aged oocytes with lower GSH:GSSG ratios (37). The GSH assay used in this study measured total GSH (GSH and GSSG) and therefore did not enable the determination of GSH:GSSG ratios. However, a higher GSH:GSSG ratio may have minimized oxidative stress induced apoptosis (21,38,39) thereby supporting better fertilization outcomes and blastocyst cell number.

While mCMRL-1066 media supported higher oocyte GSH content in late maturing MI-MII oocytes compared with HTF, 2PN fertilization was reduced and 1PN fertilization was increased in both HTF and mCMRL-1066 late MI-MII oocytes compared to IVO MII oocytes. Supplementation of mCMRL-1066 with GSH-OEt restored the 2PN rate of late MI-MII oocytes to levels observed in IVO MII oocytes and, in the case of the 5 mM GSH-OEt treatment, may be related to higher oocyte GSH content. The differential effects of 3 mM and 5 mM GSH-OEt on total oocyte GSH content and their similar beneficial effects suggest

our understanding of primate oocyte GSH requirements and utilization during maturation is incomplete.

Blastocyst development was equivalent in each of the late maturing IVM treatment groups irrespective of the oocyte GSH content. As the exact timing of polar body extrusion in late maturing MI-MII oocytes is unknown it is possible that the detrimental effects of *in vitro* oocyte ageing and delayed fertilization negatively affected embryonic development (37,40). Assessment of blastocyst cell number indicated that blastocysts derived from late maturing MI-MI oocytes were comparable to IVO MII derived blastocysts. However, as GSH-OEt did not confer the same benefit on TCN and ICM cell number as observed for the early maturing MI-MII oocytes the possibility exists that MI-MII oocytes are developmentally compromised during extended IVM.

The results of this study suggest that the poor fertilization and embryo development observed for *M. fascicularis* MI oocytes derived from ovarian stimulated cycles and IVM in HTF is related to media formulation and can be overcome with the use of a complex media such as mCMRL-1066. While fertilization and blastocyst development of *M. fascicularis* oocytes was equivalent to IVO derived oocytes when early maturing MI-MII oocytes were cultured in a complex medium, this improvement was not associated with changes to oocyte total GSH content. However, the addition of the GSH donor, GSH-OEt, further enhanced oocyte maturation rates and blastocyst cell numbers in early MI-MII oocytes and, GSH content and fertilization outcome of late maturing MI-MII oocytes. Taken together these results indicate that exogenous GSH supplementation of IVM medium results in complex effects on oocyte biology, most of which are beneficial to the oocyte in terms of oocyte fertilization and developmental potential. Further studies will be required to ascertain if the benefits of GSH-OEt supplementation may be useful for clinical application of human MI oocyte IVM when few MII oocytes are retrieved during fertility treatment.

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Figure 1.

Metaphase II (MII) oocyte intracellular glutathione content (GSH; pmol/oocyte) level of *Macaca fascicularis* MI oocytes *in vitro* matured for 18-20 h. Comparison of HTF (n=12), mCMRL-1066 (n=40) and mCMRL-1066 medium supplemented with 3 mM and 5mM glutathione ethyl ester (GSH-OEt; n=47 and 44 respectively). Data are expressed as mean $\pm$ s.e.m. for oocyte GSH content (5-12 replicates). IVO; GSH content of *in vivo* derived germinal vesicle stage (GV; n=32), metaphase I (MI; n=35) and metaphase II (MII; n=44) oocytes at time of collection (5-7 replicates). Bars with different letters are significantly different ($p < 0.05$). Data analyzed using ANOVA and post-hoc Tukey test.

Maturation and fertilization outcome of *M. fascicularis* metaphase II oocytes derived from MI oocytes *in vitro* matured for 4-6 hours

Table 1

Treatment	Maturation Rate n (%)		Fertilization Outcome n (%)			
	MII	2PN	IPN	>2PN	FF	Total Oocytes
IVO *		297 (68.6) ^{ac}	18 (4.2) ^d	8 (1.8) ^d	110 (25.4) ^e	433
HTF	39/99 (39.4) [#]	18 (51.4) ^a	9 (25.7) ^e	0 (0.0) ^d	8 (22.9) ^{ef}	35
mCMRL-1066	36/172 (20.9) [▯]	27 (90.0) ^b	1 (3.3) ^d	0 (0.0) ^d	2 (6.7) ^{df}	30
GSH-OEt 3mM	52/153 (34.0) [#]	33 (78.6) ^{bc}	2 (4.8) ^d	1 (2.4) ^d	6 (14.3) ^{def}	42
GSH-OET 5mM	57/193 (29.5) ^{#▯}	41 (82.0) ^{bc}	3 (6.0) ^{df}	0 (0.0) ^d	6 (12.0) ^f	50

* Fertilization outcome of matched sibling *in vitro* matured (IVO) MII oocytes. Different symbols within columns and different letters within rows indicate significant differences (p<0.05). 2PN; 2 pronuclei, ≥1 polar body, 1PN; 1 pronuclei, ≥1 polar body, >2PN; ≥2 pronuclei, ≥1 polar body, FF; failed fertilization, nil pronuclei, 1 polar body. Maturation data from 8-18 replicates. Fertilization data from 5-19 replicates. Maturation and fertilization data expressed as a proportion of total oocytes and differences were compared using Chi-square or Fisher Exact test.) IVO; *in vitro* matured oocytes. GSH-OEt; mCMRL-1066 with indicated supplement of glutathione ethyl ester.

Table 2

Blastocyst rate, total cell number and cell allocation of *M. fascicularis* blastocysts derived from *in vivo* matured MII oocytes and MI oocytes *in vitro* matured for 4-6 h.

Treatment	Blastocyst Rate n (%)	TCN	ICM cells	TE cells	ICM:TE
IVO *	124/399 (31.1) ^a	141.29 ± 14.52 ^a	38.69 ± 4.92 ^a	107.97 ± 12.21	2.95 ± 0.29
HTF	2/31 (6.5) ^b	ND	ND	ND	ND
mCMRL-1066	11/30 (36.7) ^a	140.10 ± 7.34 ^a	38.30 ± 4.68 ^a	101.80 ± 6.56	2.86 ± 0.36
GSH-OEt 3mM	11/42 (26.2) ^a	153.61 ± 34.32 ^{ab}	31.11 ± 3.50 ^a	122.50 ± 32.82	3.96 ± 0.92
GSH-OEt 5mM	20/47 (42.6) ^a	204.81 ± 22.39 ^b	60.75 ± 6.34 ^b	143.31 ± 20.91	2.45 ± 0.41

* Blastocyst rate of matched sibling *in vivo* matured (IVO) MII oocytes. Early MI-MII (4-6 h) blastocyst data from 5-9 replicates. Data are expressed as a proportion of total inseminated oocytes and differences were compared using Chi-square or Fisher Exact test. Blastocyst cell data from n=5-8 blastocysts. Values are mean ± s.e.m. Different letters within columns are significantly different (p < 0.05). IVO; *in vivo* matured oocytes. GSH-OEt; mCMRL-1066 with indicated supplement of glutathione ethyl ester. Data analyzed using ANOVA and post-hoc Tukey test.

Maturation, fertilization and blastocyst development of *M. fascicularis* metaphase II oocytes derived from MI oocytes *in vitro* matured for 18-20 hours.

Table 3

Treatment	Maturation Rate n (%)		Fertilization Outcome n (%)				Blastocyst Rate n (%)	
	MII		2PN	1PN	>2PN	FF	Total Oocytes	
IVO *			131 (70.1) ^a	10 (5.3) ^c	3 (1.6) ^c	43 (23.0) ^{de}	187	52/168 (31.0) [#]
HTF	41/47 (87.2) ^{#a}		6 (37.5) ^b	6 (37.5) ^b	0 (0.0) ^c	4 (25.0) ^{bce}	16	1/11 (9.1) ^a
mCMRL-1066	103/134 (76.9) [#]		24 (45.3) ^b	14 (26.4) ^{ab}	3 (5.7) ^c	12 (22.6) ^{ae}	53	5/53 (9.4) ^a
GSH-OEt 3mM	88/100 (88.0) ^a		21 (67.7) ^{ab}	2 (6.5) ^{ce}	0 (0.0) ^c	8 (25.8) ^e	31	5/26 (19.2) ^{#a}
GSH-OET 5mM	122/136 (89.7) ^a		39 (68.4) ^a	5 (8.8) ^{ce}	0 (0.0) ^c	13 (22.8) ^e	57	4/56 (7.1) ^a

* Fertilization and blastocyst rate of matched sibling *in vivo* matured (IVO) MII oocytes. Different symbols within columns and different letters within columns and rows indicate significant differences (p<0.05). 2PN; 2 pronuclei, ≥1 polar body, 1PN; 1 pronuclei, ≥1 polar body, >2PN; ≥2 pronuclei, ≥1 polar body, FF; failed fertilization, nil pronuclei, 1 polar body. Maturation data from 10-19 replicates. Fertilization data from 4-9 replicates. Blastocyst data from 3-8 replicates. Data are expressed as a proportion of total oocytes and differences were compared using Chi-square or Fisher Exact test.). IVO; *in vivo* matured oocytes. GSH-OEt; mCMRL-1066 with indicated supplement of glutathione ethyl ester.