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Improved Developmental Competence of Swampbuffalo Oocytes Matured in the Presence of Cysteamine

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Abstract The study examined the effect of cysteamine addition on the acquisition of swamp buffalo oocytes developmental competence in vitro and on the intracytoplasmicglutathione level. In a dose dependent manner, results showed significant improvement on the maturation, penetration and MPN formation of oocytes cultured with 50 µM cysteamine. Also, improved cleavage and development to the blastocyst stage were observed in oocytes matured with cysteamine addition (23.0%) versus the control (11.1%). Similarly, the intracytoplasmic glutathione level improved in oocytes cultured with cysteamine versus the control though remained lower compared to in vivo matured oocytes. Overall, the results suggest that an increase in the intracellular glutathione level of swamp buffalo oocytes as induced by cysteamine during maturation provided the oocytes with a favorable intracellular condition to support maturation, fertilization and the subsequent embryonic development in vitro.

Keywords: cysteamine, embryos, glutathione, maturation, pronuclei

Introduction

The failure of most in vitro matured and fertilized oocytes to develop normally is attributable to cytoplasmic deficiencies to support pronuclei formation and successive cleavage to implantation stage of embryo development (Rose and Bavister, 1992; Sawai *et al*., 1997). During the development and maturation of the oocytes in the ovary, the glutathione (GSH) content increases as the oocyte approaches the time of ovulation. This accumulation of GSH in the ooplasm is necessary as the oocyte prepares for fertilization (De Matos *et al*., 1996). GSH is responsible for sperm nuclear decondensation after fertilization and its antioxidant activity protects the cells from the damaging effects (oxidative stress) of free oxygen radicals generated during metabolism causing developmental arrest at the earliest period of embryonic gene expression.

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Cysteamine supplementation to in vitro maturation medium increases intracellular GSH levels resulting to improved maturation and fertilization rates (Takahashi *et al.,* 1993; De Matos *et al.,* 1995). In addition, maturation medium supplementation with cysteine or cysteamine increased the GSH content of denuded bovine oocytes thus increasing the efficacy of oocytes having few cumulus cells in a system without coculture (De Matos *et al.,* 1997). Depletion of GSH during oocyte maturation was reported to reversibly block the decondensation of MPN and pronuclear apposition during fertilization (Sutovsky and Schatten, 1997). In swamp buffalo studies, the low incidence of 1st cleavage and blastocyst formation rate indicate that deficiencies in the ooplasm do occur (Suzuki *et al*., 1992; Ocampo *et al.,* 1997). Whether these observations could be correlated to GSH deficiencies in *in vitro* cultured **SB** oocytes remained unclear, thus this study.

Materials and methods

Oocyte collection and maturation in vitro

Bubaline ovaries were collected at a local abattoir and transported to the laboratory within 5 hr in sterile physiological saline solution (0.9 % NaCl, w/v) supplemented with antibiotics (0.1 mg/ml Penicillin and 500 ug/ml Streptomycin) maintained at a temperature of 35-37 ºC. The follicular oocytes were aspirated from 3-5 mm follicles using an 18- gauge needle attached to a 10- ml disposable plastic syringe. The follicular aspirates were pooled in a test tube maintained at 37 ºC in a water bath. After 5 min, part of the follicular fluid was decanted and the sediment searched for cumulus-oocyte complexes (COCs) using a stereomicroscope at low magnification. Oocytes with intact, unexpanded cumulus cell investments and with evenly granulated ooplasm were selected for use in the experiments. Selected COCs were washed twice in Hepes buffered TCM-199 with Earles salt supplemented with 10 % fetal calf serum (FCS). The COCs were then placed in droplets of maturation medium consisting of TCM-199 + 10 % FCS + 5.5 mg/ml Na pyruvate, 0.02 IU/ml FSH, 1 μg/ml estradiol-17 β, 10 ng/ml EGF, 50 μg/ml gentamycin sulfate with or without cysteamine addition.

Sperm preparation

Straws of locally processed frozen semen from the Sperm Processing Unit of the Philippine CarabaoCenter at Central Luzon State University Ranch in Digdig, Carranglan, Nueva Ecija were thawed at 39 ºC water bath for 15 sec and washed twice with fertilization medium (Tris- buffered medium; Berger, 1990) containing 1 mg/ml BSA (Initial fractionation by heat shock, Sigma, st. Louis, MO, USA). After the final wash, the sperm was re-suspended at 1 x 10^7 sperm/ml in fertilization medium with caffeine (5 mM) and BSA (10 mg/ml), and pre-incubated for 1 hr at 39 ºC; 95 % air atmosphere in a tightly capped test tube.

In vitro fertilization

After 22-24 hr of maturation, the oocytes were denuded free of cumulus cells by pipetting and transferred to the fertilization medium (10-15 oocytes/90 µl drops) under mineral oil. A portion of the pre-incubated sperm suspension (10 µl) was added to give a final sperm concentration of 1 x 10^6 sperm/ml. The motility rate of sperm during insemination was 70 % or more. After 6 hr of sperm-oocyte coincubation, the oocytes were recovered and denuded free of extra sperm cells attached to the zonapellucida by using a finely drawn pipette. Subsequently, the oocytes were washed twice with TCM-199 medium + 10% FCS before transferring to culture droplets.

Cell counting

Blastocyst stage embryos were collected and subjected to a staining protocol (Thouas *et al.*, 2001) with modification. Briefly, blastocyst were washed in PBS-PVP, then placed in 1 ml of Hoechts working solution (0.75 ml of 2.3% Na citrate dehydrate solution; 0.25 ml of ethanol; 10 ul of Hoechts 33342 stock solution of 1 mg/ml concentration dissolved in ethanol) in an Effendorf tube, wrapped in aluminum foil and stored in the refrigerator for at least 24 hr. Subsequently, the blastocyst were recovered and washed in glycerol, mounted on a glass slide, flattened in glycerol by a cover slip to a level where all nuclei appeared at the same focal plane and examined by using a fluorescent microscope (Eclipse E-600; Nikon) under ultraviolet light. A digital image of each embryo was taken and the total cells (both inner cell mass and trophectoderm) counted.

Assay of GSH

After culture of oocytes from each maturation time endpoints (6 hr, 12 hr, 18 hr, 24 hr) in the presence or absence of cysteamine supplementation, the oocyte samples were assayed for GSH. Firstly, the cumulus cells were removed completely from the oocytes by pipetting through a narrow-bore micropipette in 0.5 M PBS-PVA (phosphate buffered saline-polyvinyl alcohol) and washed (3x) with PBS-PVA. 5 µl of PBS-PVA containing 30 oocytes was transferred to a 1.5 ml Eppendorf tube, and then 5 µl 1.25 M phosphoric acid was added that ruptured the oocytes. Tubes containing ruptured oocytes were either stored frozen (-20 °C) until use or assayed immediately for GSH concentration using the DTNB-GSSG reductase recycling assay test. Briefly, around 700 µl of 0.33 mg/ml NADPH in assay buffer (0.2 M sodium phosphate buffer containing 10 mM EDTA, pH 7.2), 100 µl of 6 mM 5,5- dithiobis-2-nitrobenzoic (DTNB) in assay buffer, and 190 µl of water were added into the tube with the oocytes. After mixing the solutions, 10 µl glutathione reductase (266 unit/ml) was added to initiate the reaction. The formation of 5-thio-2-nitrobenzoic acid (TNB) was followed continuously at an Absorbance of 412 nm using a UV Spectrophotometer connected to a recorder and recorded for 5 min (every 30 sec). The amount of GSH is determined from a standard curve assayed under the same conditions in which GSH equivalents present are plotted against the rate of change of absorbance. The values were reported in GSH equivalents (e.g., nmoles/oocyte).

Experimental

In *Experiment1*, COCs were cultured in maturation medium with or without cysteamine addition at various concentrations (e.g., 10 µM, 50 µM, 100 µM) to determine its effect on the maturation, fertilization and eventual MPN formation. After 22 hr of culture, the oocytes were subjected to IVF. Thereafter, 15 hr post insemination, the oocytes were fixed using aceto-ethanol (1:3, v/v) solution and stained with 1 % aceto-orcein for evaluation using phase-contrast microscopy. Oocytes with 1st polar body were regarded to have completed the 1st meiosis/nuclear maturation, whereas oocytes with male and female pronuclei (cytoplasmic maturation), 2nd polar body extrusion and a detached sperm tail in the ooplasm were classified as fertilized.

In *Experiment 2*, the developmental competence of resulting zygotes post IVF of oocytes matured in the presence (50 µM) or absence of cysteamine up to the blastocyst stage was determined by culturing in 100 µl droplets of TCM-199 + 20% calf serum + cumulus cell monolayer. Embryonic development was monitored every 2 days up to the 8th day of culture.

In *Experiment 3*, the intracytoplasmic GSH content of the oocytes cultured at various time points (eg., 12 hr, 18 hr, 24 hr) was determined in maturation medium with or without cysteamine supplementation (50 uM). Immediately aspirated oocytes at GV stage (0 hr) and those from preovulatory follicles (more than 10 mm in diameter) were also analyzed for their GSH.

Statistical analysis

The maturation, fertilization, MPN formation and cleavage rates expressed in percentage were averaged for each replicated treatments. Comparison among treatment means were analyzed using one way ANOVA with Tukey's honest significant difference at 5% level.

Results

In experiment 1, a total of 564 COCs were cultured *in vitro* and used for in vitro fertilization to determine the effect of cysteamine addition on the nuclear maturation, penetration and MPN formation rates. The nuclear maturation (90.7%) and penetration (86.2%) rate was highest in oocytes cultured in the maturation condition supplemented with 50 µM cysteamine. The MPN values obtained in the control (58.3 %) vs oocytes cultured with 10 µM cysteamine (57.1 %) supplementation did not differ. Likewise, the MPN values of oocytes cultured with 50 µM (57.1 %) and 100 µM (81.3 %) cysteamine addition did not differ but was greater compared to the control and those with 10 µM cysteamine supplementation (Table 1).

Table 1. Effect of cysteamine addition on the maturation, penetration and MPN formation of SB oocytes

Cysteamine addition (µM)	**No. of oocytes (%)**			
	Inseminated	Matured	Penetrated	MPN formation
0	158	134 (84.8)^{ab}	96 (60.7)^{a}	56 (58.3)^{a}
10	132	96 (72.7)^{b}	70 (53.0)^{a}	40 (57.1)^{a}
50	130	118 (90.7)^{a}	112(86.2)^{b}	84 (75.0)^{b}
100	144	122 (84.7)^{ab}	96 (66.7)^{a}	78 (81.3)^{b}

^{a,b} Differ significantly (P<0.05)

In experiment 2, a total of 362 COCs were used for IVF. The proportion of cleaved embryos from oocytes matured with cysteamine (50 µM) supplementation was higher versus the control. Similarly, the developmental progression of these embryos to the compacted morula and blastocyst stage was higher versus the control (Table 2). Likewise, the total cell count of resulting

blastocyst stage embryos was higher versus the control although showed no difference.

Table 2. Effect of cysteamine addition on cleavage and development to the blastocyst stage

No. of oocytes (%)	Total				
Cysteamine addition	Inseminated	2- to 8-	Compacted morula	Blastocyst	Cell count
-	180	54 (30.0)[a]	4(2.2) [a]	20 (11.1)[a]	102.3 ± 8.7
+	182	92 (50.5)[b]	27(15.0)[b]	42 (23.0)[b]	110.7 ± 12.5

[a,b] Values differ significantly (P<0.05)

In experiment 3, 30 GV stage and 35 in vivo matured oocytes from pre-ovulatory follicles were analyzed for their GSH content. Similarly, a total of 300 oocytes that were distributed and cultured at each time point (12 hr, 18 hr and 24 hr) in maturation condition with or without cysteamine addition were analyzed (represented by 50 oocytes/treatment). The GSH level of oocytes cultured in maturation medium without cysteamine addition were 0.002 nm, 0.003 nm, 0.004 nm and 0.005 nm/oocyte at 0 hr, 12 hr, 18 hr and 24 hr of culture, respectively. Similarly, the GSH level of oocytes cultured in maturation medium with cysteamine addition were 0.007 nm, 0.0138 nm and 0.0143 nm/oocytes at 12 hr, 18 hr and 24 hr of culture, respectively. In vivo matured oocytes had an average GSH level of 0.0185 nm/oocyte (Fig. 1).

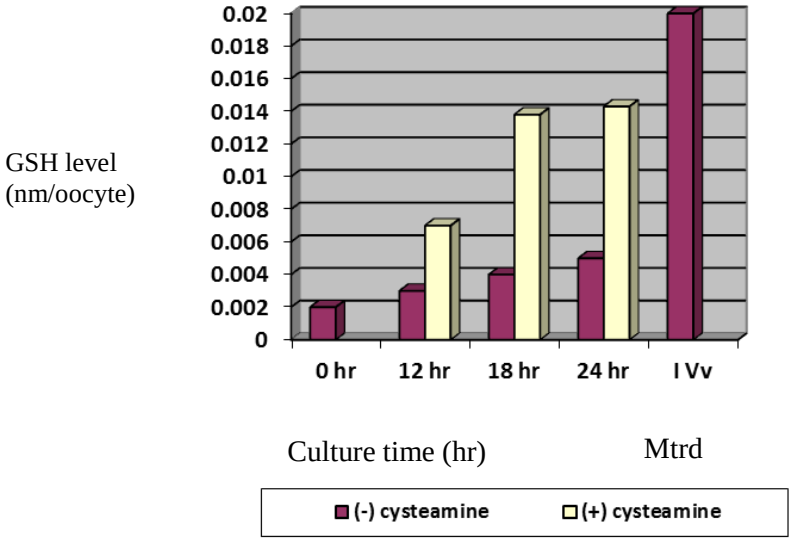

Figure 1. Intracytoplasmic glutathione levels of SB oocytes cultured in the presence or absence of cysteamine

Discussion

In this study, we have shown that SB oocytes matured in maturation medium with 50 µM or 100 µM cysteamine significantly improved the transformation efficiency of sperm head nucleus into MPN during fertilization. This acquired efficiency of the oocytes could well be correlated to the elevated intracytoplasmic GSH level of at least 0.0143 nm in oocytes matured in maturation condition supplemented with cysteamine. In contrast, 10 µM cysteamine was found insufficient in translating the needed GSH for efficient MPN formation of penetrated oocytes. Also, it is interesting to note that preovulatory oocytes had the highest GSH values upon verification, confirming further that in vivo matured oocytes are better endowed with endogenous molecules within their cytoplasm in time for fertilization and therefore have better developmental potential than in vitro matured oocytes. Based on these findings, we can conclude that sufficient GSH is paramount in inducing complete sperm decondensation in synchrony with oocyte activation to ensure the MPN formation of fertilizing sperm head. That insufficient GSH synthesis in the oocyte contributes to incomplete sperm head decondensation resulting to asynchronous development of oocyte and sperm nuclei. The results concur with previous observations in porcine and bovine that cysteamine enhances the cysteine-mediated GSH synthesis when present in the maturation medium (Grupen *et al*., 1995; De Matos *et al*., 1995), that intracellular GSH is a critical part of cytoplasmic maturation that prepare the oocytes for fertilization, activation and preimplantation embryo development (Yoshida *et al.*, 1992; 1993; Funahashi *et al.*, 1994; Eppig, 1996). It plays an important role in reducing the disulfide-rich-protamines in the sperm nucleus (Perreault, 1984; 1988) and in order for the sperm DNA to participate in embryonic development, it must be unpacked in the oocyte at the time of fertilization (Zirkin *et al*., 1985; 1989; Perreault, 1990). The reduction of sperm nuclear disulfide bonds that form during epididymal maturation is the first step in the induction of sperm nuclear decondensation.

The ability of the oocytes to support sperm head decondensation and MPN formation depends on the maturational stage of the oocytes. In this study, data taken showed that the GSH level of SB oocytes changed during maturation, and that the addition of cysteamine as a supplement in the medium promotes GSH synthesis. This observation is consistent with other reports that GSH level changes during maturation (Perreault et al., 1988). Furthermore, since GSH synthesis during in vitro maturation of SB oocytes requires exogenous compound, the decline within 24 hr in the GSH level of oocytes cultured in medium without cysteamine addition could reflect a low rate of GSH synthesis in relation to the rate of GSH use.

On the other hand, the proportion of presumptive zygotes that cleaved initially was significantly higher for oocytes matured with cysteamine. Further culture of these embryos to droplets with cumulus cell monolayer has demonstrated that the developmental competence to the blastocyst stage of SB embryos is acquired during maturation. We have shown that early- stage embryos resulting from oocytes matured in conditions with cysteamine addition developed to the morula and/or blastocyst stage better than the control. The total cell count of these embryos was also higher than those taken from without cysteamine addition indicating a more improved cell activity. Previous studies have shown that cysteamine addition to the maturation medium increases the efficiency of in vitro blastocyst production from immature bovine oocytes accompanied by an increased in intracellular GSH level (Takahashi *et al.*, 1993; De Matos *et al.*, 1995).

The specific role of GSH on SB embryogenesis could be better understood by incorporating thiol compounds (eg., cysteamine) in the culture system using simple- defined medium. In mammalian oocytes, the pool of GSH is synthesized during the 1st meiosis (Perreault *et al.*, 1984; 1988) assuring the reduction of disulfide bonds in the sperm nucleus thus promoting its decondensation into the MPN during fertilization (Wiesel and Schultz, 1981). Therefore, supplementation of the culture medium with thiol compounds that would enhance GSH synthesis seemed practical in improving protocol for the in vitro production of buffalo embryos.

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