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Effects of glutathione on oocyte and embryo development in *in vitro* fertilization

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ABSTRACT

Objective: To evaluate the effect of glutathione (GSH) supplementation in maturation and adaptation media on oocyte development, embryo quality, and oocyte viability after vitrification.

Methods: The GSH concentrations were classified into four groups (0, 0.5, 1.0, and 1.5 mM) which were added to the maturation medium. The maturation process was conducted *in vitro* for 24 h. Following maturation, oocytes were fertilized with Bali bull semen for 5–6 h and then cultured for 48 h. The morphological quality of oocytes matured with GSH addition and the vitrification method used was evaluated. Parameters assessed included maturation rate, fertilization rate, embryo development, post-vitrification oocyte morphology, and quality of post-vitrification oocytes with GSH added to the adaptation medium.

Results: The addition of GSH to the maturation medium significantly improved oocyte quality and embryo development ($P < 0.05$). Specifically, adding 1.5 mM GSH increased the percentage of oocytes reaching metaphase II from 57.6% without GSH oocytes to 79.0% with 1.5 mM GSH, two-pronuclei fertilization from 47.0% to 72.7%, embryo development from 37.1% to 57.2%, morula formation from 14.6% to 33.7%, and blastocyst formation from 8.1% to 23.8%. Additionally, the survival rate of oocytes post-vitrification increased to 75% with GSH supplementation.

Conclusions: The addition of 0.5–1.5 mM of GSH to the maturation and adaptation media significantly enhanced the metaphase II stage, fertilization rates, cleavage rates, and the survival of oocytes after vitrification. Among the concentrations of 1.5 mM was the most effective in increasing oocyte development and maintaining oocytes viability post-vitrification.

KEYWORDS: Bali cattle; Embryo; Glutathione; Oocyte; Vitrification

1. Introduction

The adoption of cryopreservation technology presents promising avenues for addressing female infertility. Cryopreservation methods

Key Points

Question: Does glutathione (GSH) supplementation in maturation and adaptation media improve oocyte quality and embryo development?

Findings: GSH supplementation significantly improved oocyte quality and embryo development. Particularly, GSH at concentration of 1.5 mM significantly enhanced oocyte maturation, fertilization rates, and post-vitrification survival.

Meaning: These findings have broader implications for enhancing reproductive health and fertility, offering pathway to optimize oocyte cryopreservation techniques and improve overall reproductive efficiency in *in vitro* fertilization.

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primarily include slow freezing and vitrification, with the latter recognized as a rapid freezing technique. Vitrification is the preferred method for preserving mammalian oocytes and embryos due to its superior efficacy and reduced risk of cellular damage during the freezing process[1]. Oocyte cryopreservation plays a crucial role in treatment on female infertility and is considered to have greater clinical significance compared to embryo freezing[2]. Additionally, the cryopreservation of oocytes can serve as an initial phase in the conservation of bovine embryos[3].

Oocytes are particularly vulnerable to freezing temperatures, with their biological functions ceasing entirely at extremely low temperatures (-196°C). Despite these harsh conditions, cell viability and functional integrity can be prevered over extended periods under controlled conditions[4]. Freezing protocols aim to minimize cellular damage by incorporating techniques such as dehydration and freezing point depression[5]. The vitrification process requires the use of cryoprotectants to maintain oocyte quality during freezing[6]. It is essential to select non-toxic cryoprotectants to ensure oocyte preservation, facilitate fertilization, and support subsequent embryo development[3].

Culturing oocytes or embryos *in vitro* under manipulated environmental conditions can elevate reactive oxygen species (ROS) levels leading to cellular damage[5]. Introducing antioxidants into the culture media effectively counteracts ROS levels[7]. Antioxidants are compounds that stabilize free radicals by providing electrons, thereby preventing a chain reaction of free radicals[7]. Glutathione (L- γ -glutamyl-L-cysteinyl-glycine; GSH) is an antioxidant that can be incorporated into maturation media[8]. GSH plays a crucial role in protecting cells from ROS-induced damage and supports protein and DNA synthesis by regulating redox status[5]. Moreover, GSH aids in the elimination of xenobiotics and peroxides, serving as a primary antioxidant within cells[9]. GSH levels increase during oocyte maturation in the ovary, reaching a peak at the metaphase II (M II) stage[5].

In bovine species, fertilization rates tend to remain relatively low, highlighting the need for improvements in the developmental competence of cryopreserved oocytes[10]. Incorporating an antioxidant-rich maturation medium during *in vitro* oocyte maturation has been shown to improve oocyte quality[11]. Therefore, this study aims to determine the optimal concentration of GSH to be added to the maturation medium, ensuring its effectiveness in enhancing oocyte development and post-freezing viability in Bali cattle oocytes.

2. Materials and methods

2.1. Study period and location

This study was conducted from February 2023 to December 2023. Ovaries were collected from the Tamangapa slaughterhouse in



Figure 1. Oocyte of Bali cattle before maturation (magnification 100 $\times$).

Makassar, Indonesia. *In vitro* fertilization (IVF) were performed and evaluated at the Laboratory for *In vitro* Embryo Production, Hasanuddin University. Semen was sourced from the Regional Artificial Insemination Center (RAIC) in South Sulawesi. The research involved supplementing the maturation and adaptation media with varying concentrations of GSH: P0=0 mM, P1=0.5 mM, P2=1.0 mM, and P4=1.5 mM.

2.2. Oocyte collection and selection

The study utilized 205 pairs of Bali cattle ovaries obtained from slaughterhouses and transported to the laboratory in 0.9% NaCl solution. Oocyte were collected by the slicing method and selected under an Olympus SZ51 microscope. Only oocytes classified as grades A and B (Figure 1) were chosen based on established criteria[12].

2.3. Oocyte maturation

The selected oocytes were matured in medium containing M199 medium (Gibco by Life Technologies, USA) with 0.3% bovine serum albumin (BSA), 10 IU/mL pregnant mare serum gonadotrophin (PMSG) (Intergonan, Intervet Deutschland GmbH), 10 IU/mL human chorionic gonadotrophin (hCG) (Chorulon, Intervet International BV Boxmeer-Holland), and 50 $\mu\text{g/mL}$ gentamycin (Sigma-Aldrich, USA), supplemented with varying concentrations of GSH (0.0 mM, 0.5 mM, 1.0 mM, and 1.5 mM). Maturation medium was dispensed in drops (80 $\mu\text{L/drop}$), each containing 10-15 oocytes, and covered with mineral oil (Sigma Chemical Co. St. Louis MAU, USA). Oocyte maturation was conducted in a 5% CO_2 incubator at 38.5°C for 24 h[13].

2.4. IVF

Semen was collected from a single bull, thawed at 37°C for

20 s, and centrifuged at 600×g for 5 min (repeated twice) to separate spermatozoa from plasma. Spermatozoa were then resuspended in fertilization media to a final concentration of 1.5×10^6 cells/mL[14]. Four drops of fertilization medium (80 µL/drop) were placed in a petri dish, covered with mineral oil (Sigma Chemical Co. St. Louis MO, USA), and allowed to equilibrate for 30 min. Matured oocytes were then transferred into the fertilization medium and incubated at 38.5 °C in 0.5% CO₂ for 24 h.

2.5. *In vitro* cultures

Following 5-6 h of fertilization, oocytes were washed twice in Charles Rosenkrans (CR1aa) culture medium, containing 0.4 mM sodium pyruvate, 5 mM lactate, 5% calf serum, 20 µg/mL streptomycin sulphate. They were then transferred into 80 µL drops of CR1aa culture medium supplemented with 5 mg/mL BSA, 2.5% fetal bovine serum (FBS), and covered with mineral oil (Sigma Chemical Co. St. Louis MO, USA). The oocytes were cultured in an incubator at 38.5 °C in 0.5% CO₂[15]. Embryo division was assessed 48 h after culture initiation, and embryos were further cultured for a total of 5 days before final evaluation.

2.6. *Vitrification of oocytes*

Oocyte vitrification was performed using a closed straw system with vitrification media comprised 3% BSA, 15% dimethyl sulfoxide (DMSO), 15% ethylene glycol (EG), and 0.5 M sucrose. The oocytes were equilibrated in an equilibration medium containing 3% BSA and 10% EG for 3-5 min. Following equilibration, the oocytes were loaded into 0.35 mL straw[6]. The filling sequence for the straws was as follows: sucrose, air space, sucrose, air space, vitrification medium, air space, vitrification medium containing oocytes, air space, and finally sucrose. The open end of each straw was sealed, and the straws were cooled in liquid nitrogen vapor for 10 s. In this study, the oocytes were vitrified for 14 days before thawing.

2.7. *Thawing oocytes*

The oocytes were thawed after being stored in liquid nitrogen. The liquid nitrogen straw was allowed to reach room temperature for 6 s before being immersed in a water bath at 37 °C for 30 s. The straw was gently agitated to mix the solution, and the contents were then transferred to a thawing medium containing 3% BSA and 0.25 M sucrose. Subsequently, the oocytes were placed in an adapted medium comprising 3% BSA, gentamicin, and GSH at concentrations of 0.0 mM, 0.5 mM, 1.0 mM, and 1.5 mM. After 30 min recovery in phosphate-buffered saline (PBS), the oocytes were evaluated for viability. Surviving oocytes displayed a typical round shape and showed no signs of lysis, shrinkage, swelling, or darkening[6].

2.8. *Evaluation of oocytes quality*

Mature and fertilized oocytes were evaluated by first removing the cumulus cells using a 0.25% hyaluronidase enzyme. The oocytes were then placed on glass slides coated with paraffin and vaseline, covered with a cover glass. The preparation was fixed in a solution containing acetic acid and ethanol (1:3) for 5 days. One hour before staining, the slides were soaked in absolute ethanol and subsequently stained with 2% aceto-orcein for 5 min. The staining agent was rinsed off with 25% acetic acid, and oocyte morphology was observed under a light microscope (Olympus CX23, Japan).

Mature oocytes were categorized into distinct stages: germinal vesicle (GV), metaphase I (MI), anaphase I (AI), telophase I (TI), and MII. Oocytes exhibiting a discernible nuclear membrane were classified as being in the germinal vesicle stage and considered immature. Those displaying a metaphase plate along with the presence of the first polar body were categorized as being in the MII stage and deemed mature. Furthermore, oocytes were classified as being in an intermediate stage if the nuclear membrane was no longer visible, encompassing the MI, AI, and TI stages of maturation, between the germinal vesicle and MII stages[16].

The assessment of IVF capability involved examining the formation and quantity of pronuclei in fertilized oocytes. Typically, fertilized oocytes display the formation of two pronuclei (male and female, 2PN) within the cytoplasm. In contrast, polyspermy-fertilized oocytes contain more than 2PN. Fertilization rates were categorized into three criteria: total fertilization rate, normal fertilization rate, and polyspermy rate. The total fertilization rate is determined by comparing the number of fertilised oocytes to the total number of oocytes. The normal fertilization rate is calculated by comparing the number of oocytes that formed 2PN with the total number of fertilised oocytes. Meanwhile, polyspermy is calculated as the ratio of oocytes with more than 2PN to the total number of fertilized oocytes[17].

2.9. *Statistical analysis*

Data were analyzed using SPSS ver. 25.0 (IBM Corp., Armonk, NY, USA) and presented as mean±standard deviation (mean±SD). Significant differences between treatments were assessed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. A $P < 0.05$ was considered statistically significant.

2.10. *Ethical approval*

This research was approved by the Animal Ethics Commission of Hasanuddin University, Makassar, Indonesia (approval number: 404/UN4.6.4.5.31/PP36/2022, dated May 10, 2022).

3. Results

3.1. Maturation rate of Bali cattle oocytes with the addition of GSH to the maturation media

The maturation stage rates are illustrated in Figure 2. The findings indicate that the inclusion of GSH in the maturation media significantly influenced the maturation levels in Bali cattle ($P<0.05$), as depicted in Table 1. A total of 103 oocytes matured without the addition of GSH, with the following results: (6.3±2.3)% ($n=7$) oocytes in the GV stage, (12.9±4.5)% ($n=14$) in the GVBD stage, (23.2±5.2)% ($n=24$) in the M I stage, and (57.6±7.8)% ($n=58$) in the M II stage. For oocytes treated with 0.5 mM GSH with a total of 100 oocytes were resulting in (5.6±1.9)% ($n=7$) in the GV stage, (11.9±1.7)% ($n=12$) in the GVBD stage, (15.2±4.5)% ($n=16$) in the M I stage, and (67.2±9.7)% ($n=65$) in the M II stage. The total number of oocytes matured with 1.0 mM GSH was 120 oocytes, resulting in (3.9±0.4)% ($n=4$) in the GV stage, (8.0±1.6)% ($n=14$) in the GVBD stage, (10.5±3.9)% ($n=14$) in the M I stage, and (77.7±6.6)% ($n=92$) in the M II stage. Meanwhile, a total of 113 oocytes matured with the addition of 1.5 mM GSH, resulting in (2.7±0.9)% ($n=3$) in the GV

stage, (7.9±4.7)% ($n=9$) in the GVBD stage, (10.4±2.9)% ($n=12$) in the M I stage, and (79.0±8.4)% ($n=89$) in the M II stage.

The results indicated that the addition of 0.5 mM to 1.5 mM GSH to the maturation media significantly enhanced the level of oocyte maturation to the M II compared to the control group ($P<0.05$). The percentage of oocytes reaching the M II stage without GSH was significantly lower than that of oocytes matured with the addition of GSH. This findings suggests that GSH positively influences the *in vitro* maturation of Bali cattle oocytes. The addition of 1.5 mM GSH to the maturation media appeared to be the most effective for increasing the maturation rate of Bali cattle oocytes (Table 1).

3.2. The fertilization rate of Bali cattle oocytes with the addition of GSH to the maturation media

Fertilized oocytes were characterized by the presence of more than one pronucleus. While, unfertilized oocytes are identified by having either one pronucleus or no pronucleus, as illustrated in Figure 3. The results indicated that the addition of GSH to the maturation media significantly improved the fertilization rate in Bali cattle, as evidenced by a reduction in the number of degenerated oocytes:

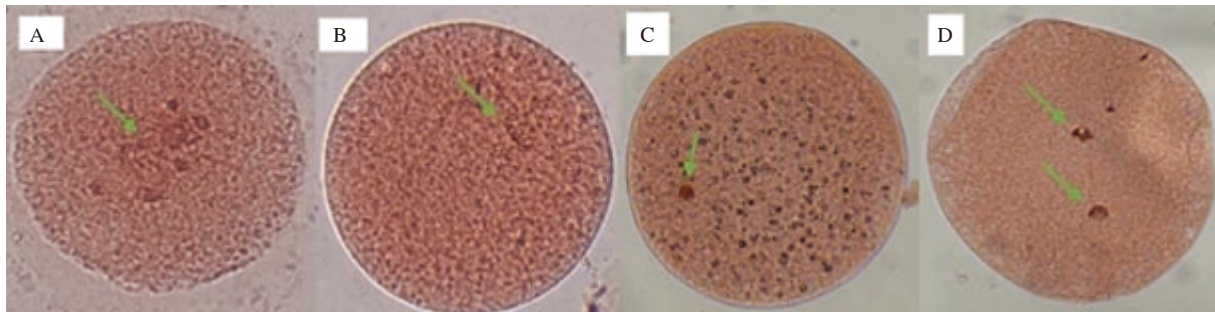


Figure 2. Oocyte maturation: germinal vesicle (GV) (A), germinal vesicle break down (GVBD) (B), metaphase- I (M I) (C), and metaphase- II (M II) (D) (magnification 200×). Arrow in Panel A: Indicates the intact germinal vesicle (GV), representing the oocyte at the initial stage of maturation. Arrow in Panel B: Marks the process of germinal vesicle breakdown (GVBD), a critical transition phase toward further oocyte maturation. Arrow in Panel C: Highlights the arrangement of chromosomes at the metaphase- I (M I) stage, indicative of the first meiotic division. Arrows in Panel D: Point to chromosomes aligned at the metaphase- II (M II) stage, signifying the completion of oocyte maturation prior to fertilization.

Table 1. The maturation rate of Bali cattle with the addition of glutathione to the maturation media.

Treatment	Number of oocytes (n)	Maturation rate [$n(\%)$]			
		GV	GVBD	M I	M II
P0	103	7 (6.3±2.3)	14 (12.9±4.5) ^b	24 (23.2±5.2) ^b	58 (57.6±7.8) ^a
P1	100	7 (5.6±1.9)	12 (11.9±1.7) ^{ab}	16 (15.2±4.5) ^a	65 (67.2±9.7) ^b
P2	120	4 (3.9±0.4)	14 (8.0±1.6) ^a	14 (10.5±3.9) ^a	92 (77.7±6.6) ^b
P3	113	3 (2.7±0.9)	9 (7.9±4.7) ^a	12 (10.4±2.9) ^a	89 (79.0±8.4) ^b

P0: 0.0 mM of GSH, P1: 0.5 mM of GSH, P2: 1.0 mM of GSH, and P3: 1.5 mM of GSH. Different letters within the same variable indicate significant difference ($P<0.05$). GSH: glutathione; GV: germinal vesicle; GVBD: germinal vesicle break down; M I: metaphase- I; M II: metaphase- II.

Table 2. The fertilization rate of Bali cattle with the addition of glutathione to the maturation media.

Treatment	Number of oocytes	Fertilization rate [$n(\%)$]			
		1 PN	2 PN	>2 PN	Degerated
P0	105	23 (22.1±4.8)	49 (47.0±3.6) ^a	9 (7.7±4.7)	24 (23.3±3.8) ^c
P1	97	23 (23.9±5.2)	55 (57.4±8.8) ^b	7 (6.5±4.5)	12 (12.2±4.5) ^b
P2	108	24 (21.4±6.2)	70 (65.7±5.7) ^c	4 (4.4±2.7)	10 (9.5±5.0) ^{ab}
P3	91	16 (17.6±3.4)	66 (72.7±4.3) ^d	4 (4.4±3.4)	5 (5.5±2.8) ^a

Note: P0: 0.0 mM of glutathione (GSH), P1: 0.5 mM of GSH, P2: 1.0 mM of GSH, and P3: 1.5 mM of GSH. Different letters within the same variable indicate significant difference ($P<0.05$). PN: pronuclei.

Table 3. The development of Bali cattle embryos with the addition of glutathione to maturation media.

Treatment	Number of oocytes	Development of embryo [n(%)]		
		Cleavage rate	Morula	Blastocyst
P0	108	40 (37.1±3.7) ^a	16 (14.6±4.3) ^a	9 (8.1±2.1) ^a
P1	120	55 (45.4±3.8) ^b	27 (23.4±4.9) ^b	18 (14.4±2.6) ^b
P2	94	48 (51.6±5.8) ^c	29 (30.9±3.7) ^c	20 (21.2±1.0) ^c
P3	102	58 (57.2±2.4) ^d	34 (33.7±5.0) ^c	24 (23.8±3.9) ^c

Note: P0: 0.0 mM of glutathione (GSH), P1: 0.5 mM GSH, P2: 1.0 mM GSH, and P3: 1.5 mM GSH. Different letters within the same variable indicate significant difference ($P<0.05$).

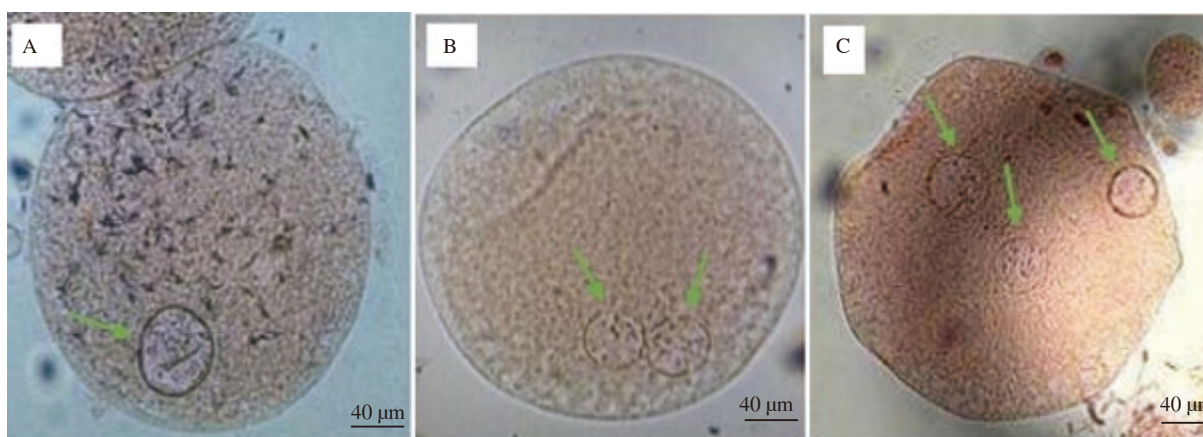


Figure 3. The development of Bali cattle oocytes after fertilization: the appearance of one-pronucleus (1PN) (A), two pronuclei (2PN) (B), and oocyte pronuclei (>2PN) (C). Scale bar=40 µm. Arrow in Panel A: Indicates the single pronucleus (1PN) observed in the oocyte, representing successful fertilization at the initial stage. Arrows in Panel B: Highlight the presence of two pronuclei (2PN), which signify the syngamy phase, where male and female pronuclei are visible before fusion. Arrows in Panel C: Point to the appearance of multiple pronuclei (>2PN), indicating abnormal fertilization or polyspermy.

(23.3±3.8)% ($n=24$) without GSH compared to (5.5±2.8)% ($n=5$) with GSH ($P<0.05$) (Table 2).

In total, 105 oocytes matured without GSH: 23 had 1 pronucleus (1PN) (22.1±4.8)%, 49 had 2 pronuclei (2PN) (47.0±3.6)%, 9 exhibited more than 2 pronuclei (>2PN) (7.7±4.7)%, and 24 showed degeneration (23.3±3.8)%. With the addition of 0.5 mM GSH, a total of 97 oocytes were fertilized: 23 had 1 pronucleus (1PN) (23.9±5.2)%, 55 had 2 pronuclei (2PN) (57.4±8.8)%, 7 had more than 2 pronuclei (>2PN) (6.5±4.5)%, and 12 showed degeneration (12.2±4.5)%.

In the group with 1.0 mM GSH, a total of 108 oocytes were matured, resulting in (21.4±6.2)% ($n=24$) had 1 pronucleus (1PN), (65.7±5.7)% ($n=70$) had 2 pronuclei (2PN), (4.4±2.7)% ($n=4$) had more than 2 pronuclei (>2PN), and (9.5±5.0)% ($n=10$) exhibiting degeneration. In addition, in the group with 1.5 mM GSH, a total of 91 oocytes were matured, with (17.6±3.4)% ($n=16$) had 1 pronucleus (1PN), (72.7±4.3)% ($n=66$) had 2 pronuclei (2PN), (4.4±3.4)% ($n=4$) had more than 2 pronuclei (>2PN), and (5.5±2.8)% ($n=5$) showed degeneration. These findings demonstrate that the addition of GSH not only increased fertilization rate but also significantly reduced degeneration (Table 2).

3.3. The embryo development rate with the addition of GSH to the maturation media

The rate of embryo cleavage in Bali cattle significantly increased

with the addition of GSH to the maturation medium, with the enhanced being proportional to the concentration of GSH. The development stages of Bali cattle embryos are shown in Table 3.

In the control group, a total of 108 oocytes cultured post-fertilization, resulting in 40 oocytes (37.1±3.7)% reaching the cleavage stage, 16 developing into morulas (14.6±4.3)%, and 9 forming blastocysts (8.1±2.1)%. A total of 120 oocytes were cultured, and with the addition of 0.5 mM GSH to the maturation media, 55 embryos reached the cleavage stage (45.4±3.8)%, 27 developed into morulas (23.4±4.9)%, and 18 formed blastocysts (14.4±2.6)%. A total of 94 mature oocytes were cultured, and with the addition of 1.0 mM GSH were matured and divided into 48 reaching the cleavage stage (51.6±5.8)%, 29 developing into morulas (30.9±3.7)%, and 20 forming blastocysts (21.2±1.0)%. Additionally, 102 fertilized and cultured oocytes with the addition of 1.5 mM were divided into 58 reaching the cleavage stage (57.2±2.4)%, 34 developing into morulas (33.7±5.0)%, and 24 forming blastocysts (23.8±3.9)% (Table 3).

The results showed that the addition of GSH to the maturation media significantly affected the rates of cleavage, morula formation, and blastocyst development in Bali cattle ($P<0.05$) (Figure 3). Furthermore, the findings indicated that the addition of 1.00 mM GSH in the maturation medium did not result in a statistically difference compared to 1.5 mM GSH in supporting the development of morula and blastocysts in Bali cattle.

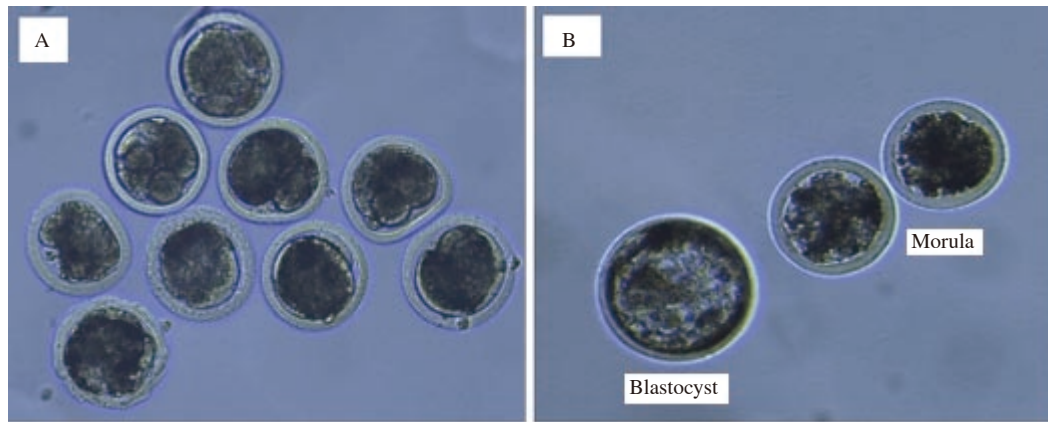


Figure 4. Embryo development after two days of culture (a) and five days of culture (magnification 100×).

Table 4. Morphological evaluations of oocytes after thawing with the addition of glutathione to maturation media.

Treatment	Number of oocytes	Morphology [n(%)]			
		Oocyte survival	Fracture of pellucida	Shrinkage of cytoplasmic	Lysis of cytoplasmic
P0	74	39 (51.2±7.0) ^a	10 (14.4±5.1) ^b	15 (20.8±6.7) ^b	10 (13.6±5.2)
P1	88	56 (63.89±6.7) ^b	8 (8.0±2.0) ^a	16 (16.5±4.0) ^{ab}	11 (11.7±4.7)
P2	95	66 (69.3±9.9) ^b	5 (4.6±2.6) ^a	11 (12.3±3.8) ^a	13 (10.7±5.0)
P3	81	58 (72.2±8.6) ^b	7 (4.9±1.1) ^a	7 (12.3±3.8) ^a	7 (10.6±3.7)

Note: P0: 0.0 mM of glutathione (GSH), P1: 0.5 mM of GSH, P2: 1.0 mM of GSH, and P3: 1.5 mM of GSH. Different letters within the same variable indicate significant difference ($P<0.05$).

Table 5. Morphological evaluations of oocytes after thawing with the addition of glutathione to the adapted media.

Treatment	Number of oocytes	Morphology [n(%)]			
		Oocyte survival	Fracture of pellucida	Shrinkage of cytoplasmic	Lysis of cytoplasmic
P0	106	56 (54.0±11.3) ^a	16 (15.3±4.6) ^c	18 (17.9±5.9) ^b	16 (14.3±4.3) ^b
P1	90	61 (68.3±9.1) ^b	11 (12.6±4.8) ^{bc}	12 (13.9±7.0) ^{ab}	6 (6.6±3.7) ^a
P2	66	47 (72.2±8.6) ^b	4 (4.9±1.3) ^a	6 (8.3±3.2) ^a	9 (13.9±4.3) ^b
P3	78	57 (73.3±6.1) ^b	7 (8.9±2.0) ^{ab}	7 (8.9±2.0) ^a	7 (8.9±2.0) ^a

Note: P0: 0.0 mM of glutathione (GSH), P1: 0.5 mM of GSH, P2: 1.0 mM of GSH, and P3: 1.5 mM of GSH. Different letters within the same variable indicate significant difference ($P<0.05$).

3.4. Morphological quality of Bali cattle oocytes after vitrification with the addition of GSH to the maturation media

The addition of GSH to the maturation media affected the quality of oocytes after vitrification. The morphology of Bali cattle oocytes after vitrification can be seen in Figure 4. A total of 74 oocytes were vitrified without the addition of GSH (P0), resulting in 39 oocytes surviving (51.2±7.0)%, 10 with zona pellucida fractures (14.4±5.1)%, 15 with cytoplasmic shrinkage (20.8±6.7)%, and 10 with cytoplasmic lysis (13.6±5.2)%. With the addition of 0.5 mM GSH (P1), 88 vitrified oocytes resulted in 56 oocytes surviving (63.9±6.7)%, 8 with zona pellucida fractures (8.0±2.0)%, 16 with cytoplasmic shrinkage (16.5±4.0)%, and 11 with cytoplasmic lysis (11.7±4.7)%. Furthermore, with the addition of 1.0 mM GSH, a total of 95 vitrified oocytes showed 66 oocytes surviving (69.3±9.9)%, 5 with zona pellucida fractures (4.6±2.6)%, 11 with cytoplasmic shrinkage (12.3±3.8)%, and 13 with cytoplasmic lysis (10.7±5.0)%. The addition of 1.5 mM GSH resulted in a total of 81 vitrified oocytes, with 58 oocytes surviving (72.2±8.6)%, 7 with zona pellucida fractures (4.9±1.1)%, 7 with cytoplasmic shrinkage (12.3±3.8)%, and 7 with cytoplasmic lysis (10.6±3.7)% (Table 4).

The results indicated that adding GSH to the maturation media

significantly improved oocyte outcomes compared to media without GSH. This improvement was demonstrated by a higher oocyte viability rate in GSH supplemented media prior to cryopreservation, in contrast to the medium without GSH ($P<0.05$) (Table 4). The addition of 0.5–1.5 mM GSH led to a 20% increase in oocyte survival relative to the control (without GSH), suggesting that GSH effectively enhances oocyte survival and viability in maturation media post-vitrification.

3.5. Morphological quality of Bali cattle oocytes after vitrification with the addition of GSH to adapted media

The addition of GSH to adapted media significantly improved the quality of oocytes after vitrification. In the control group (P0), where 106 oocytes were vitrified without GSH, the survival rate was (54.0±11.3)% ($n=56$), (15.3±4.6)% ($n=16$) with observed defects including zona pellucida fracture, (17.9±5.9)% ($n=18$) with cytoplasm shrinkage, and (14.3±4.3)% ($n=16$) with other abnormalities. In contrast, when 0.5 mM GSH was added to the vitrification medium ($n=90$), the survival rate increased to (68.3±9.1)% ($n=61$), with a reduction in zona pellucida fractures (12.6±4.8)% ($n=11$), and moderate cytoplasmic lysis (13.9±7.0)%

($n=12$), and moderate cytoplasmic lysis ($6.6\pm3.7\%$) ($n=6$).

Further improvement was observed with 1.0 mM GSH with 66 vitrified oocytes, showed a survival rate of ($72.2\pm8.6\%$) ($n=47$), with intact zona pellucida ($4.9\pm1.3\%$) ($n=4$), with cytoplasm shrinkage ($8.3\pm3.2\%$) ($n=6$), and with cytoplasm lysis ($13.9\pm4.3\%$) ($n=9$). The addition of 1.5 mM GSH to 78 vitrified oocytes resulted in a survival rate of ($73.3\pm6.1\%$) ($n=57$), with zona pellucida fracture ($8.9\pm2.0\%$) ($n=7$), with cytoplasm shrinkage ($8.9\pm2.0\%$) ($n=7$), and with cytoplasm lysis ($8.9\pm2.0\%$) ($n=7$). Overall, the addition of 0.5–1.5 mM GSH to the adapted media led to improved oocyte viability, with a nearly 20% increase in survival compared to the control group ($P<0.05$), indicating that GSH is effective in enhancing oocyte survival post-vitrification (Table 5).

4. Discussion

This study showed that increasing the concentration of GSH in the maturation media positively influenced the developmental competence of Bali cattle oocytes. Specifically, as the GSH concentration increased, the proportion of oocytes reaches the MII stage from 57.6% in the control group (without GSH) to 79.0% with the addition of 1.5 mM GSH. A previous study reported the same results in sheep, where an increase in the concentration of GSH in the maturation media led to an increase in the percentage of mature oocytes[6].

Furthermore, Malott *et al*[18] reported that a deficiency of GSH in oocytes can result in reduced oocyte competence and an increase in oxidative stress, which can compromise oocyte quality. The observed improvement in oocyte maturation with the addition of GSH may be attributed to its antioxidant properties, which help mitigate oxidative damage during the maturation process. These findings reinforce the importance of GSH as a crucial factor in enhancing the quality and developmental potential of oocytes during *in vitro* maturation.

The addition of GSH to the maturation medium resulted in a significant improvement in oocyte quality, as evidenced by the higher proportion of oocytes reaching the MII stage compared to the controls. A GSH concentration of 1.5 mM showed the highest level of MII maturity (79.0%). GSH plays an active role in protecting oocyte cells, enabling the oocytes to attain cellular maturity. Exogenous GSH has the potential to scavenge free radicals to increase the competence of oocyte development *in vitro* in bovine and porcine, particularly in oocytes lacking cumulus cells[19].

A GSH concentration of 1.5 mM showed a higher rate of oocyte fertilization in Bali cattle. The GSH antioxidant system serves as the primary endogenous defense mechanism against oxidative stress, playing a direct and active role in neutralizing ROS and maintaining the reduced (active) forms of vitamins C and E, which are essential for cellular function and protection[20]. GSH, as an intracellular antioxidant derived from the body's cells, is often referred to as the master antioxidant because it regulates the functioning of other antioxidants[21]. Its role as an intracellular antioxidant is particularly significant in the context of IVF[22], where the addition

of antioxidants like GSH has been shown to improve fertilization outcomes by protecting oocytes from oxidative damage[23]. Among antioxidants, GSH has been found to have superior efficacy in safeguarding cellular integrity, particularly in preserving DNA and RNA chain integrity within the cell nucleus. This protective action is critical for maintaining oocyte quality and fertilization competence, as free radicals can cause significant damage to genetic material, impairing fertilization and early embryonic development[24].

In mature oocytes, GSH plays a crucial role in the formation of the male pronucleus after fertilization and during the early stages of embryo development[2]. The GSH present in the oocyte cytoplasm is involved in the process of pronucleus formation, which begins with the disruption of disulfide bonds in the nuclear membrane. This disruption facilitates the subsequent initiation of chromosome decondensation in the fertilization process[25]. The ability of GSH to regulate such molecular events is essential for maintaining oocyte quality and ensuring successful early embryonic development. By modulating oxidative stress and supporting the integrity of cellular components, GSH contributes significantly to the successful formation of the pronucleus, which is crucial for the proper progression of fertilization and subsequent embryo development.

The division rate of Bali cattle oocytes supplemented with GSH was significantly higher than the 41.01% division rate reported by Damayanti *et al*[26] for oocytes without GSH. GSH affects embryo development; the higher the concentration of GSH in the maturation media, the greater the development of Bali cattle embryos. Ren *et al*[19] reported that the exogenous addition of GSH enhances the developmental competence of grade B and C oocytes, facilitating their progression to embryos. Furthermore, exogenous GSH influences intracellular GSH levels through the glutamyl- γ cycle, which plays a critical role in promoting early embryonic development in cattle[8].

The success of *in vitro* embryo culture is largely determined by the embryo's ability to reach the morula and blastocyst stages[24]. Antioxidants play a crucial role in supporting *in vitro* embryo culture. The optimal dosage of antioxidants in the culture media is a critical factor in ensuring the successful development of embryos, as it influences the balance between ROS and the cellular defense mechanisms required for healthy embryonic growth[27].

The higher the GSH concentration, the greater the survival rate of oocytes after vitrification. The addition of glutathione ethyl ester (GSH-Oet) in cattle maturation media after vitrification has the effect of maintaining mitochondrial distribution patterns, reducing cytoplasmic and mitochondrial ROS content, and increasing the percentage of embryos that develop the 8-cell stage[5]. Gunawan *et al*[6] reported that GSH supplementation improved the percentage of normal oocytes in sheep after vitrification.

The addition of 1.5 mM GSH to the maturation media resulted in the highest oocyte viability post-vitrification, with a survival rate 73.3%. This enhancement is attributed to GSH, a triol tripeptide (γ -glutamylcysteinylglycine), known as a non-protein sulphhydryl component that plays an important part in detoxification and acts as an antioxidant to maintain intracellular redox conditions. GSH

combats oxidative stress in both its reduced and oxidized form[28]. The initial condition of the oocyte before vitrification significantly affects the quality of the oocyte after the vitrification process[29].

The inclusion of GSH in the maturation medium effectively preserves oocyte quality during the vitrification process. The results showed that the metabolic pathway serves a significant role in cytoplasmic maturation. Supplementation of bovine maturation media with thiol compounds is a useful tool for increasing the efficiency of embryo production *in vitro*, considering that the increased GSH produced during maturation can be maintained during the fertilization process and remain present at the beginning of embryo culture[5]. Adding GSH at a concentration of 5 mmol/L significantly enhanced the overall antioxidant capacity of oocytes following vitrification[9]. The combination of insulin growth factor-1 (IGF-1) and GSH in the maturation media is relevant for enhancing the *in vitro* maturation process to improve oocyte cryotolerance and post-vitrification thawing[23]. The addition of murine culture media with GSH increased the freezing tolerance of embryos by reducing ROS levels, inhibiting apoptotic, and improving oxidative status[2].

The quality of the oocytes used in this study was selected based on homogenous cytoplasm and compact cumulus cells, ensuring they had similar developmental potential, and as a result, the oocyte morphology after thawing was expected to be consistent. As previously reported, the initial oocyte quality is also pivotal in influencing the oocytes's morphology after freezing[29]. The current study showed that adding of GSH to the adapted media is very important to maintain oocytes and help them survive.

Heat stress in vitrified oocytes has an impact on the oocytes[30]. GSH is a low molecular weight antioxidant that is easily synthesized and is crucial in regulating the cell cycle[19]. The addition of antioxidants to the adapted media proves that GSH can enhance the viability of Bali cattle oocytes after vitrification. Antioxidant can preserve oocyte integrity after vitrification, preventing undesired exocytosis of cortical granules[27]. The addition GSH-Oet to the maturation media enhances the cryopreservation tolerance of bovine oocytes during vitrification by preserving mitochondrial distribution patterns, reducing cytoplasmic and mitochondrial ROS levels, and improving embryo development[5].

The limitation of the current study is the specific range of GSH concentration utilized. Therefore, further research is needed to explore the effects of other concentrations and to examine longer-term outcomes of embryo development beyond the blastocyst stage. Furthermore, the study primarily assessed morphological parameters. Future research should include molecular analyses to gain a deeper understanding of mechanism through which GSH improves oocyte and embryo quality.

In conclusion, the addition of 0.5-1.5 mM of GSH to the maturation and adaptation media significantly enhanced the MII stages, fertilization rates, cleavage rates, and the survival of oocytes after vitrification. The addition of 1.5 mM to the media proved to be the most effective in increasing oocyte development and maintaining oocytes after vitrification.

Conflict of interest statement

The authors have no conflict of interest to declare.

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Data availability statement

The data of this study are available from the corresponding author upon reasonable request.

Authors' contributions

Herry Sonjaya, Erni Damayanti, and Hikmayani Iskandar designed the study and wrote the draft article. Erni Damayanti, Hasbi Hasbi, Sudirman Baco, Eka Mulyawati Kaiin, Andi Mujnisa, and Kusumandari Indah Prahesti conducted the experiments and collected the literature. Aulia Uswa Noor Khasanah, Sri Firmiaty, and Asma'ul Fitriana Nurhidayah performed the data for analysis. Rukman Pala collected the oocyte from the slaughterhouse. Daud Samsudewa, Fitra Aji Pamungkas, and Herdis Herdis assisted with the literature collection and data interpretation. All authors read and approved the final version of the manuscript.

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References

- [1] Zhou Y, Fu X, Zhou G, Jia B, Fang Y, Hou Y, et al. An efficient method for the sanitary vitrification of bovine oocytes in straws. *J Anim Sci*

- Biotechnol* 2014; **5**(1): 1-7.
- [2] Rahman NSA, Khan NAMN, Eshak Z, Sarbandi MS, Kamal AAM, Malek MA, et al. Exogenous *L*-glutathione improves vitrification outcomes in murine preimplantation embryos. *Antioxidants* 2022; **11**(11): 1-21.
- [3] Mullen SF, Fahy GM. A chronologic review of mature oocyte vitrification research in cattle, pigs, and sheep. *Theriogenology* 2012; **78**(8): 1709-1719.
- [4] Hwang IS, Hochi S. Recent progress in cryopreservation of bovine oocytes. *Biomed Res Int* 2014; **2014**: 570647.
- [5] García-Martínez T, Vendrell-Flotats M, Martínez-Rodero I, Ordóñez-León EA, Álvarez-Rodríguez M, López-Béjar M, et al. Glutathione ethyl ester protects *in vitro*-maturing bovine oocytes against oxidative stress induced by subsequent vitrification/warming. *Int J Mol Sci* 2020; **21**(20): 1-26.
- [6] Gunawan M, Nuriza N, Kaiin EM, Sjahfirdi L. The effect of glutathione antioxidant addition in maturation medium on the morphology of Garut sheep (*Ovis aries*) oocytes after vitrification. *J Phys Conf Ser* 2021; **1725**: 012062. doi: 10.1088/1742-6596/1725/1/012062.
- [7] Iskandar H, Sonjaya H, Yusuf M, Hasbi H. Effect of the addition of insulin transferrin selenium on *in vitro* maturation and fertilization of Bali cattle oocytes. *J Ilmu Tern Vet* 2019; **24**(3): 95-102.
- [8] Li F, Cui L, Yu D, Hao H, Liu Y, Zhao X, et al. Exogenous glutathione improves intracellular glutathione synthesis via the γ -glutamyl cycle in bovine zygotes and cleavage embryos. *J Cell Physiol* 2019; **234**(5): 7384-7894.
- [9] Olexiková L, Dujčíková L, Makarevich AV, Bezdrček J, Sekaninová J, Nesvadbová A, et al. Glutathione during post-thaw recovery culture can mitigate deleterious impact of vitrification on bovine oocytes. *Antioxidants* 2023; **12**(1): 1-17.
- [10] Mogas T. Update on the vitrification of bovine oocytes and *in vitro*-produced embryos. *Reprod Fertil Dev* 2019; **31**(1): 105-117.
- [11] Khazaei M, Aghaz F. Reactive oxygen species generation and use of antioxidants during *in vitro* maturation of oocytes. *Int J Fertil Steril* 2017; **11**(2): 63-70.
- [12] Saleh WM. Assessment of different methods of bovine oocytes collection, maturation and *in vitro* fertilization of abattoir specimens. *Iraqi J Vet Sci* 2017; **31**(1): 55-65.
- [13] Damayanti E, Sonjaya H, Baco S, Hasbi H. Apoptosis in Bali cattle embryo cells produced *in vitro*. *World's Vet J* 2022; **12**(4): 462-467.
- [14] Hasbi H, Iskandar H, Sonjaya H, Purwantara B, Arifiantini RI, Agil M, et al. Comparative developmental competence of *in vitro* embryos recovered from Bali cattle with normal and poor sperm motility. *Vet World* 2024; **17**(3): 593-601.
- [15] Truong TT, Gardner DK. Antioxidants increase blastocyst cryosurvival and viability post-vitrification. *Hum Reprod* 2020; **35**(1): 12-23.
- [16] Sharma A, Tiwari M, Gupta A, Pandey AN, Yadav PK, Chaube SK. Journey of oocyte from metaphase-I to metaphase-II stage in mammals. *J Cell Physiol* 2018; **233**(8): 5530-5536.
- [17] Hasbi H, Sonjaya H, Gustina S. Oocytes population and development competence of Bali cattle embryo *in vitro* with different ovarian reproductive statuses. *Trop Anim Sci J* 2022; **45**(4): 389-396.
- [18] Malott KF, Reshel S, Ortiz L, Luderer U. Glutathione deficiency decreases lipid droplet stores and increases reactive oxygen species in mouse oocytes. *Biol Reprod* 2022; **106**(6): 1218-1231.
- [19] Ren J, Hao Y, Liu Z, Li S, Wang C, Wang B, et al. Effect of exogenous glutathione supplementation on the *in vitro* developmental competence of ovine oocytes. *Theriogenology* 2021; **173**: 144-155.
- [20] Sharifi-Rad M, Anil Kumar NV, Zucca P, Varoni EM, Dini L, Panzarini E, et al. Lifestyle, oxidative stress, and antioxidants: Back and forth in the pathophysiology of chronic diseases. *Front Physiol* 2020; **11**: 1-21.
- [21] Golubkoca A, Leiva T, Snyder K, Schlegel C, Bonvicino SM, Agbaga MP, et al. Response of the glutathione (GSH) antioxidant defense system to oxidative injury in necrotizing enterocolitis Alena. *Antioxidants* 2023; **12**(7): 1385.
- [22] Nurkarimah DA, Kaiin EM, Karja NWK, Setiadi MA. The effectivity of cysteamine supplementation on improving the *in vitro* fertilization rate of sheep oocytes. *J Vet Sci* 2021; **15**: 118-122.
- [23] Pereira BA, Zangeronimo MG, Castillo-Martín M, Gadani B, Chaves BR, Rodríguez-Gil JE, et al. Supplementing maturation medium with insulin growth factor I and vitrification-warming solutions with reduced glutathione enhances survival rates and development ability of *in vitro* matured vitrified-warmed pig oocytes. *Front Physiol* 2019; **9**: 1-13.
- [24] Kurutas EB. The importance of antioxidants which play the role in cellular response against oxidative/nitrosative stress: Current state. *Nutr J* 2016; **15**(1): 1-22.
- [25] Chakravarthi S, Jessop CE, Bulleid NJ. The role of glutathione in disulphide bond formation and endoplasmic-reticulum-generated oxidative stress. *EMBO Rep* 2006; **7**(3): 271-275.
- [26] Damayanti E, Sonjaya H, Baco S, Hasbi H, Kaiin EM. Epidermal growth factor (EGF) and basic fibroblast growth factor (FGF2) profiles in follicular fluid, maturation media and embryo culture of Bali cattle. *Adv Anim Vet Sci* 2023; **11**: 1532-1539.
- [27] Yusufli MB, Çizmeci SÜ. The effect of antioxidants added to culture medium on blastocyst development rates. *Turkish J Agric Food Sci Technol* 2022; **10**: 1671-1679.
- [28] Luberda Z. The role of glutathione in mammalian gametes. *Reprod Biol* 2005; **5**(1): 5-17.
- [29] Dujčíková L, Makarevich AV, Olexiková L, Kubovičová E, Strejček F. Methodological approaches for vitrification of bovine oocytes. *Zygote* 2020; **29**(1): 1-11.
- [30] Vendrell-Flotats M, Arcarons N, Barau E, López-Béjar M, Mogas T. Effect of heat stress during *in vitro* maturation on developmental competence of vitrified bovine oocytes. *Reprod Domest Anim* 2017; **52**: 48-51.