

## THE EFFECTIVITY OF CYSTEAMINE SUPPLEMENTATION ON IMPROVING THE *IN VITRO* FERTILIZATION RATE OF SHEEP OOCYTES

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### ABSTRACT

The purpose of this study was to evaluate the effectiveness of cysteamine supplementation on the maturation medium and/or *in vitro* fertilization medium with regards to improving the normal fertilization rate of sheep oocytes, which are characterized by the formation of two pronuclei. Grade A and B oocytes were matured in medium-199 with 0.3% bovine serum albumin (BSA), 10 IU/mL follicle stimulating hormone (FSH), 10 IU/mL human chorionic gonadotrophin (hCG), and 50 µg/mL gentamicin added for 24 hours in a 5% CO<sub>2</sub> incubator at 39° C. The treatment group was divided into the following groups: a control group with no cysteamine (P1), a group with 100 µM cysteamine in the maturation medium (P2), a group with 100 µM cysteamine in the fertilization medium (P3), and a group with 100 µM cysteamine both in the maturation and fertilization medium (P4). The fertilization was carried out by incubating sperm-oocytes for 12 hours and then staining them with 1% aceto-orcein to observe the formation of a pronucleus. Normal fertilization rates obtained by each treatment group were 56.5% (P1), 57.1% (P2), 57.8% (P3), and 59.9% (P4) with no significant difference ( $P>0.05$ ) between groups. It was concluded that 100 µM cysteamine supplementation in both the maturation medium and fertilization medium was not able to increase the normal fertilization rate of sheep oocytes.

Key words: cysteamine, GSH, oocyte, pronucleus, sheep

### ABSTRAK

Tujuan dari penelitian ini adalah mengevaluasi efektivitas penambahan cysteamine pada medium maturasi dan/atau medium fertilisasi *in vitro* dalam memperbaiki tingkat fertilisasi normal oosit domba yang ditandai dengan terbentuknya dua pronukleus. Oosit grade A dan B dimatangkan dalam medium-199 yang ditambahkan 0,3% bovine serum albumin (BSA), 10 IU/mL follicle stimulating hormone (FSH), 10 IU/mL human chorionic gonadotrophin (hCG) dan 50 µg/mL gentamisin selama 24 jam dalam inkubator CO<sub>2</sub> 5% dengan suhu 39° C. Kelompok perlakuan dibagi menjadi kelompok kontrol tanpa penambahan cysteamine (P1), kelompok dengan penambahan 100 µM cysteamine dalam medium maturasi (P2), penambahan 100 µM cysteamine dalam medium fertilisasi (P3), dan penambahan 100 µM cysteamine kombinasi dalam media maturasi dan fertilisasi (P4). Fertilisasi dilakukan dengan menginkubasi sperma-oosit selama 12 jam kemudian diwarnai dengan 1% aseto-orcein untuk mengamati pembentukan pronukleus. Tingkat fertilisasi normal yang didapat oleh setiap kelompok perlakuan adalah 56,5% (P1); 57,1% (P2); 57,8 % (P3); dan 59,9% (P4) tidak menunjukkan perbedaan yang signifikan ( $P>0,05$ ) antar kelompok. Disimpulkan bahwa suplementasi 100 µM cysteamine dalam media maturasi, media fertilisasi, dan kombinasinya belum mampu meningkatkan jumlah fertilisasi normal oosit domba.

Kata kunci: cysteamine, GSH, oosit, pronucleus, domba

### INTRODUCTION

Oocytes for *in vitro* embryo production (IVEP) of livestock are usually obtained from the ovary waste of slaughterhouses. These oocytes typically have undergone puberty and exhibit a perfect oocyte ultrastructure so that they are ready to be used in IVEP (Hyttel *et al.*, 1997; Jainudeen *et al.*, 2000). The application of this biotechnology involves several important stages, including *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) (Gordon, 2003). The success of each stage of IVEP depends on a the following factors: the components of the *in vitro* culture medium, the quality of the oocytes used, and the environmental conditions during culture (Hammam *et al.*, 2010).

*In vitro* cultures are generally carried out with a level and pressure that is equivalent to the O<sub>2</sub> in the atmosphere (~20%), while the O<sub>2</sub> level in *in vivo* is usually around 5%, with the O<sub>2</sub> pressure in the follicular fluid being around 3-13% (Hashimoto *et al.*, 2000; Swain *et al.*, 2016). *In vitro* cultures with such

O<sub>2</sub> levels have been known to increase oxygen metabolism which leads to an increase in the formation of free radicals or reactive oxygen species (ROS) (Correa *et al.*, 2008; Mingoti *et al.*, 2009). An imbalance in the number of ROS can cause damage to cell structures, including the mitochondria, microtubules, deoxyribonucleic acid (DNA), and ribonucleic acid (RNA) (Takahashi *et al.*, 2000). The increase in ROS production due to an increase in oxygen metabolism in *in vitro* culture conditions can be reduced by adding antioxidant compounds to the culture medium (Livingston *et al.*, 2009). Glutathione (GSH) is one of the intracellular antioxidants produced by oocytes through a *de novo* synthesis process during the maturation process (de Matos *et al.*, 1996). GSH plays an important role in reducing free radicals; protecting cellular from oxidative stress; as a reservoir of cysteine; modulating various cellular processes, including DNA and protein synthesis, processes related to microtubules, cell immune function, cell proliferation during the embryonic process (Lu, 2009), and helping to decondensate the nucleus of

spermatozoa cells during the formation of the pronucleus (Yoshida *et al.*, 1993).

When an oocyte undergoes nuclear maturity, GSH reaches its highest concentration and the gap junction will be cut off due to the secretion of the hyaluronidase enzyme by the cumulus cells so that cysteine cannot be transported back into the oocyte (de Matos and Furnus, 2000; Sutton *et al.*, 2003). If the concentration of GSH achieved during maturation is not optimal, it will cause an accumulation of ROS which results in aging and apoptosis (Agarwal *et al.*, 2005; Combelles *et al.*, 2009). The components of the basic culture media used in the IVM, and IVF processes are not equipped with antioxidants; however, in medium-199 there is cysteine which is the main ingredient of GSH production with very small concentrations of 0.6 M and 108.3 M cystine. In a basic medium, IVF (Suzuki *et al.*, 2000) does not contain cysteine as a component. Conversely, if oxygen levels are high in an *in vitro* culture environment, cysteine will easily oxidize into cystine. Thus, a substance is needed that can keep cysteine in a stable condition during the IVM and IVF processes.

Cysteamine is an amino thiol ( $\text{HSCH}_2\text{CH}_2\text{NH}_2$ ) that is formed in the body from the degradation of coenzyme A during the formation of pantothenic acid, which eventually hydrolyzes into cysteamine and pantothenic acid. Cysteamine is able to increase the production of GSH during the maturation process by reducing cystine to cysteine (Gasparrini *et al.*, 2003; Zhou *et al.*, 2008). Supplementation of thiol components such as cysteamine can break cystine disulfide bonds into cysteine and cysteine-cysteamine disulfide forms, thereby triggering cysteine uptake and GSH synthesis. Cysteamine in low concentrations can increase cysteine transport into cells for GSH synthesis, but in high concentrations it can reduce the activity of the enzyme glutathione peroxidase which is important in catalyzing GSH into disulfide bonds (Besouw *et al.*, 2013). A number of animal studies have shown that the addition of 100  $\mu\text{M}$  cysteamine to an IVM medium can increase the number of fertilized oocytes in goats (An *et al.*, 2018), pigs (Bing *et al.*, 2002), and cattle (de Matos *et al.*, 2002a). At the time this study was conducted, the effectiveness of the addition of cysteamine in the fertilization medium was still unknown; therefore, this study was aimed at examining the increase in the competence of sheep oocytes after the addition of cysteamine to the maturation and fertilization medium.

## MATERIALS AND METHODS

### Ovary Collection

Ovaries of pubescent goats and sheep, which were characterized by the presence of a corpus luteum in one of the ovaries, obtained from slaughterhouses were rinsed with 0.9% NaCl supplemented with 0.1 mg/mL streptomycin (Sigma-Aldrich) and 100 mg/mL penicillin (Sigma-Aldrich, St. Louis, MO, USA). The ovaries were placed in a thermos containing the same solution used for rinsing at a temperature of 34°-36° C. The ovaries were then brought to the laboratory in less than 3 hours after excision.

### Oocyte Collection and *In vitro* Maturation

The oocytes were collected by a slicing technique using a size 21 blade. The oocyte collection was carried out in sterile petri dishes containing a phosphate buffered saline (PBS) solution supplemented with 0.3% bovine serum albumin (BSA) (Sigma-Aldrich, Inc., P-4687), 100 IU/mL penicillin, and 0.1 mg/mL streptomycin before being washed three times. The oocytes collected were evaluated and selected under a stereomicroscope. Grade A and B oocytes with a minimum of 3 layers of compact cumulus cells and homogeneous cytoplasm were selected for the maturation process.

Oocyte maturation was carried out using a static culture system in which oocytes were matured in microdroplets containing 100  $\mu\text{L}$  of a maturation medium containing 15–20 oocytes, which was then covered with mineral oil (Sigma, USA). The maturation medium used was basic medium-199 (TCM-199) (Gibco, Grand Island, NY, USA) supplemented with 0.3% BSA (Sigma, USA), 10 IU/mL follicle stimulating hormone (FSH) (Vetoquinol N.-A inc, Lavaltrie, Quebec, Canada), 10 IU/mL human chorionic gonadotrophin (hCG) (Kyoritsu Seiyaku, Tokyo, Japan) and 50  $\mu\text{g}/\text{mL}$  gentamycin (Sigma, USA). The oocyte maturation was carried out in a 5%  $\text{CO}_2$  incubator at 39° C for 24 hours (Zhu *et al.*, 2018).

### Spermatozoa Preparation and *In vitro* Fertilization

The oocyte fertilization was carried out using frozen Garut sheep semen from the Lembang Artificial Insemination Center, Bandung. The frozen sheep semen was thawed in a water bath at 37° C for 30 seconds. The semen was then put into a sterile centrifuge tube containing 8 mL of fertilization medium (Suzuki *et al.*, 2000) and centrifuged at 1800 G for 5 minutes. The supernatant portion from the centrifugation was discarded and the spermatozoa precipitate was diluted again with a fertilization medium to obtain a final concentration of  $5 \times 10^6$  spermatozoa/mL. A mixture of spermatozoa and fertilization media was made in the form of 100  $\mu\text{L}$  drop and covered with mineral oil (Sigma-Aldrich, Inc., M-8410). The matured oocytes were washed twice in the fertilization and then transferred into a drop and incubated for 12-14 hours in a 5%  $\text{CO}_2$  incubator at 39° C. After 12-14 hours of incubation, the oocyte fertilization rate was evaluated.

### Evaluation of Fertilization Success

The fertilized oocytes were denuded with cumulus cells, with the help of 0.25% hyaluronidase enzyme (Sigma, USA), by pipetting repeatedly using a pipette with the diameter adjusted to the size of the oocyte. The denuded oocytes were then placed in a PBS drop on a cover glass which had paraffin and Vaseline pads at each of the four corners of the glass, then they were fixed to both sides of the paraffin and Vaseline pads. The preparations were put into a fixation solution containing acetic acid and ethanol (1:3) for 48-72 hours. The preparations were then stained with 1%

aceto orcein for 5 minutes, then the dye was rinsed with 25% acetic acid. The pronucleus (PN) observation was performed using a phase contrast microscope

Determination of the rate of *in vitro* fertilization ability was carried out based on the formation and number of PN. Total fertilized oocytes are oocytes that have two or more PNs. Normal fertilization is characterized by the formation of 2PN, while oocytes with more than 2PN are categorized as polyspermy.

### Experimental Design

The treatments in this study were divided into four groups based on cysteamine supplementation in an IVM medium and/or IVF medium. The concentration of cysteamine used was 100  $\mu$ M, which was in reference to the study by An *et al.* (2018) which used goats as experimental animals. The control group was the group without cysteamine supplementation referred to as P1, and P2 was the treatment group with cysteamine supplementation in the maturation medium. P3 was the group with cysteamine supplementation on the fertilization medium, and P4 was the group with cysteamine supplementation on both the maturation and fertilization mediums.

### Data Analysis

The *in vitro* oocyte fertilization rate data is presented in percentage form and was statistically analyzed using analysis of variance (ANOVA) at a 95% significance level, followed by Duncan's multiple range test (DMRT) (Steel and Torrie, 1993).

## RESULTS AND DISCUSSION

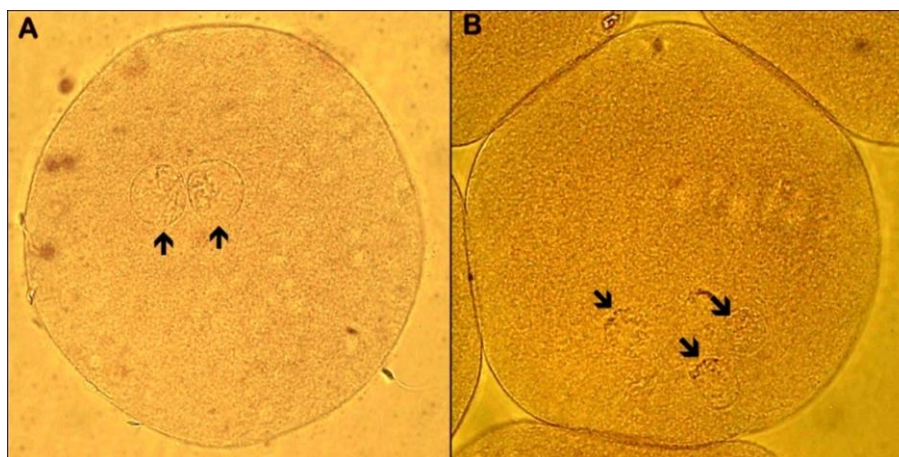
From this study, it was found that the total fertilization rate in the groups with the addition of 100  $\mu$ M cysteamine at P2 (71.8 $\pm$ 0.8), P3 (73.5 $\pm$ 0.8), and P4 (73.6 $\pm$ 1.3) were not significantly different ( $P < 0.05$ ) from the control group P1 (68.5 $\pm$ 1.3). Furthermore, there was no significant difference ( $P > 0.05$ ) between the groups with the addition of cysteamine compared to the control group. These results show that the addition of 100  $\mu$ M cysteamine in the maturation medium, fertilization medium, and combined medium did not increase the normal fertilization number of sheep oocytes as presented in Table 1.

The success of the IVF process is usually judged by the formation of two or more pronuclei. Meanwhile, normal fertilization is characterized by the formation of two pronuclei and the oocytes that have more than two pronuclei are categorized as polyspermy oocytes (Elder and Dale, 2003) as in Figure 1. This is because oocytes with normal fertilization have the opportunity to develop further into embryos. In the fertilization process, GSH can reduce disulfide bonds in the zona pellucida (ZP) which allows for spermatozoa to penetrate more easily into the oocyte (Takeo and Nagata, 2011). In addition, Sutovsky *et al.* (2000) stated that GSH reduces the disulfide bonds in the tail of spermatozoa so that tail severance can occur, and this in turn initiates the formation of the pronucleus. After formation of the pronucleus occurs, GSH reduces the disulfide bonds in the protamine, which is then

**Table 1.** Sheep oocyte fertilization rate with supplementation of 100  $\mu$ M cysteamine in IVM medium and/or IVF medium

Groups	Treatment		Oocyte number	Pronucleus formation rate (% mean $\pm$ SEM)		
	100 $\mu$ M cysteamine			Normal 2 PN (%)	Polyspermy >2PN (%)	Total fertilized (%)
	IVM medium	IVF medium				
P1	-	-	84	48(56.5 $\pm$ 3.4)	10(12.0 $\pm$ 2.5)	58(68.5 $\pm$ 1.3)
P2	+	-	92	54(57.1 $\pm$ 4.5)	12(14.7 $\pm$ 4.1)	66(71.8 $\pm$ 0.8)
P3	-	+	90	53(57.8 $\pm$ 2.9)	13(15.7 $\pm$ 3.5)	66(73.5 $\pm$ 0.8)
P4	+	+	83	50(59.9 $\pm$ 3.4)	11(13.7 $\pm$ 3.2)	61(73.6 $\pm$ 1.3)

The mean in the same column with different superscripts shows a significant difference ( $P < 0.05$ ). += With 100  $\mu$ M cysteamine, -= Without 100  $\mu$ M cysteamine, 2PN= Two pronucleus, >2PN= More than two pronucleus



**Figure 1.** Pronucleus (PN) formation after *in vitro* fertilization of sheep oocyte. A= Oocyte with two pronucleus (2 PN); B= Oocyte with more than two pronucleus (>2 PN). 1% aceto orcein 200 $\times$  magnification

replaced by histones as a condition for decondensation (Mukhopadhyay *et al.*, 2008). If there is no reduction of the disulfide bond by GSH then the formation of the pronucleus does not occur (Sutovsky and Schatten, 1997).

The results of this study showed that the addition of 100  $\mu\text{M}$  cysteamine in the maturation medium, fertilization medium and combined mediums did not significantly increase the normal fertilization rate in all treatments. There are two possibilities that might have caused no significant difference in all of these treatments at a dose of 100  $\mu\text{M}$ . The first one being that the addition of cysteamine at a dose of 100  $\mu\text{M}$  was not able to increase the number of mature oocytes for fertilization. This is in line with the research of de Matos *et al.* (2002b), who stated that there was no increase in oocyte maturation with the addition of cysteamine at a dose of 100  $\mu\text{M}$  in sheep. A significant increase in oocyte development and GSH production during maturation was only achieved at a dose of 200  $\mu\text{M}$ . This is presumably because the addition of cysteamine at this concentration is not able to produce an optimal GSH concentration to support the formation of the pronucleus. This result is in line with studies on mice oocytes (Chen *et al.*, 2005) and bovine oocytes (Balasubramanian and Rho, 2007), which stated that the addition of 100  $\mu\text{M}$  cysteamine had no significant effect on fertilization rates.

The second hypothesis is that the addition of cysteamine to the media at different stages of culture affects the formation of GSH through the conversion of cysteine to cystine. In this study, the increase in normal fertilization rates in both the control and treatment group did not show a significant difference. It is possible that this event is caused by the GSH that is formed to support normal fertilization in the maturation medium, which is supported by the availability of the main ingredients for forming GSH, namely cysteine and cystine. This is in accordance with the opinion of Besouw *et al.* (2013) and Lu (2013) who stated that cysteamine would break the disulfide bonds of cystine into the form of cysteine and cysteine-cysteamine disulfide which are the main ingredients for the formation of GSH. In this study, the TCM-199 medium used had a cysteine component with a concentration of 0.6 M and cystine 108.3  $\mu\text{M}$ .

Similar to the results on the maturation medium, 100  $\mu\text{M}$  cysteamine supplementation in the IVF medium did not show a significant difference in normal fertilization rates compared to the other groups. It is suspected that the addition of cysteamine in the fertilization medium did not play a role in increasing the normal fertilization rate. The reason for this occurrence may be related to the fact that the peak of the oocyte GSH formation process occurs during the nuclear maturation process, and this gradually decreases at the beginning of the fertilization process until it enters the early stages of embryonic development (de Matos and Furnus 2000;

Zuelke *et al.*, 2003). In addition, the synthesis of GSH through the addition of cysteamine requires

certain substances as the main ingredients to facilitate its formation, namely cysteine and cystine. Meanwhile, the fertilization medium used (Suzuki *et al.*, 2000) did not contain these two components at all. This reinforces the notion that the addition of cysteamine to the fertilization medium does not support the development of normal fertilization rates.

The addition of 100  $\mu\text{M}$  cysteamine supplementation to both the IVM medium and IVF medium did not affect the normal fertilization rate of sheep oocytes. These results indicate that the GSH required for the formation of a normal pronucleus is dependent upon the basic medium of *in vitro* maturation.

## CONCLUSION

Based on the study conducted, it can be concluded that the addition of 100  $\mu\text{M}$  cysteamine was not able to effectively increase the normal fertilization rate of sheep oocytes *in vitro*.

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