

# Cryopreservation of Indonesian native chicken semen by using dimethyl sulfoxide and various level of ethylene glycol as cryoprotectants

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**Abstract.** Khaeruddin, Junaedi, Hastuti. 2020. Cryopreservation of Indonesian native chicken semen by using dimethyl sulfoxide and various level of ethylene glycol as cryoprotectants. *Biodiversitas* 21: 5718-5722. Imported purebred chickens are becoming more popular and a regular staple in Indonesia. Therefore, it is necessary to strengthen conservation efforts to preserve Indonesian chickens, one of which is by means of sperm cryopreservation. This study aimed to determine the effects of the addition of DMSO and different concentrations of ethylene glycol to a Ringer's lactate egg yolk (RLY)-or coconut water egg yolk (CWY)-based extender on the quality of frozen-thawed Indonesian chicken sperm. This study was used nine Indonesian native roosters about 20 months of age. The semen extenders used in this study were RLY + DMSO 7%, RLY + ethylene glycol 3%, RLY + ethylene glycol 5%, RLY + ethylene glycol 7%, CWY + DMSO 7%, CWY + ethylene glycol 3%, CWY + ethylene glycol 5% and CWY + ethylene glycol 7%. Liquid semen was packaged in 0.25 mL straw, then cooled at 5°C for 2 hours, frozen at 5 cm above liquid nitrogen for 10 minutes, following stored in a liquid nitrogen container for 24 hours. The semen straws were thawed at 37°C for 30 seconds. Statistical analysis for multiple comparisons was performed as a completely randomized design with eight treatment levels and seven replications. The results showed that there were no differences in sperm motility, recovery rate, and abnormality between extenders after the freeze-thaw process. Whereas, RLY + DMSO 7% was the highest sperm viability.

**Keywords:** Cryopreservation, DMSO, ethylene glycol, Indonesian chicken sperm

## INTRODUCTION

Indonesia has different types of chickens (*Gallus gallus domesticus*) that can be found across several islands. However, imported purebred chickens are becoming more popular and a regular staple in Indonesia. It is, therefore, necessary to strengthen conservation efforts to preserve Indonesian chickens. Cryopreservation is one of the effective methods of preserving chicken sperm. According to Bhattacharya and Prajapati (2016), the main objective of cryopreservation is to create a dehydrating environment for preserving cells. One factor that can determine the success of cryopreservation is the composition of the semen extender, as it affects the quality of the sperm after the freeze-thaw process. Coconut water is an inexpensive extender and contains essential nutrients, namely sugar and several minerals, needed by chicken sperm during storage. Ringer's lactate has been proven to be a good semen extender due to its isotonic properties and has been widely studied in Indonesian chicken semen stored at 5°C.

Penetrative cryoprotectants are among the ingredients added to extenders to protect sperm from the harmful effects of freezing. According to Bhattacharya and Prajapati (2016), penetrative cryoprotectants have a molecular mass of fewer than 100 kDa and penetrate inside the cells and maintain moisture during freeze-drying. Glycerol, dimethylacetamide, and dimethyl sulfoxide (DMSO) were cryoprotectants that had been reported in the

freezing of Indonesian native chicken sperm (Junaedi et al. 2016a,b, 2017, 2019). DMSO is an organosulfur derivative. Its molecular formula is  $(\text{CH}_3)_2\text{SO}$ . This colorless solution can be able to dissolve both polar and non-polar compounds (Bhattacharya and Prajapati 2016). Another cryoprotectant that could potentially be used in chicken sperm cryodiluent is ethylene glycol (EG). Ethylene glycol has a molecular weight of  $62.1 \text{ g mol}^{-1}$ , lower than the molecular weight of DMSO, which is  $78.3 \text{ g mol}^{-1}$  (Leibo and Pool 2011). According to Bhattacharya and Prajapati (2016), ethylene glycol alters the hydrogen bonding when it mixes with water. After mixing with 40% water and 60% ethylene glycol, the freezing point of the mixture would depress and the mixture becomes incapable of forming crystalline substances. This property of ethylene glycol makes it the most effective candidate for cryoprotection.

Previous studies have proven the effectiveness of ethylene glycol in mammalian sperm freezing. Silva et al. (2012) found that the progressive motility of sheep sperm after thawing was higher at 3-5% ethylene glycol when compared to acetamide. Meanwhile, Chen et al. (2017) stated that Ethylene glycol had the same ability as glycerol in maintaining motility, acrosome integrity, and mitochondrial membrane potential in apes' sperm.

The use of ethylene glycol resulted in higher motility of the dog's sperm after thawing compared to glycerol (Rota et al. 2006). Other reports indicate that the increase of sperm quality and pregnancy rate in buffaloes occurred

when glycerol was replaced by ethylene glycol in an extender containing egg yolk (Swelum et al. 2011). Ethylene glycol has been reported to be used in cryodiluent of muscovy duck sperm, but it is not as good as the use of DMSO (Gerzilov 2010). However, DMSO and ethylene glycol are suitable for cryopreservation of semen of Venda chickens (Mphaphathi et al. 2016). However, the concentration of cryoprotectant needs to be investigated. According to Bhattacharya and Prajapati (2016), the optimization of cryoprotectants as elevated concentration could cause cytotoxicity. This study aimed to determine the effects of the addition of DMSO and different concentrations of ethylene glycol to a Ringer's lactate egg yolk-or coconut water egg yolk-based extender on the quality of frozen-thawed Indonesian chicken sperm.

## MATERIALS AND METHODS

### Collection and evaluation of the semen

This study was used nine Indonesian native roosters about 20 months of age. The chickens were kept in individual cages measuring 40 x 50 x 70 cm<sup>3</sup> and given a total of 150 grams of chicken feed daily and drinking water was given by *ad libitum*. Semen was collected in the morning at 07.30 using a massage technique based on Burrows and Quinn (1936). Semen was obtained by placing the left hand on the back of the chicken and massaging around the cloaca with two fingers until the papilla protruded. This protrusion of the papilla was followed by the ejaculation of fresh semen. The semen that was collected was immediately evaluated for characteristics both macroscopically (volume, color, viscosity, and pH) and microscopic (sperm concentration, sperm mass movement, sperm motility, sperm viability, and sperm abnormality).

Sperm concentration was calculated using a hemocytometer *Neubauer chamber* under a 160x magnification microscope, and mass movement was evaluated by observing a drop of semen on a glass slide and evaluated under a binocular light microscope (Boeco, Germany) with 160x magnification microscope. The motility of the sperm was determined by viewing some semen on a glass slide with a cover glass under a 400x magnification microscope. To determine the viability and abnormality of the sperm, some semen was stained with eosin-nigrosin (Merck, KgaA, Darmstadt Germany) on a glass slide, homogenized, placed on a glass slide, and dried on a heating table for 5 seconds. Ten fields of view were determined under a 640x magnification microscope by calculating the percentage of sperm that did not absorb eosin-nigrosin (viability) and the percentage of abnormal morphology (abnormality). The entire procedure for evaluating the quality of the sperm was carried out according to Arifiantini (2012).

### Extender preparation

The basic extender was made from Ringer's lactate (PT. Widatra Bakti, Indonesia) which contained 1.55 g of sodium lactate, 3 g of sodium chloride, 0.15 g potassium chloride, and 0.1 g of calcium chloride in 500 mL sterile water, with osmolarity 274 mOsm L<sup>-1</sup>, or natural young coconut water which was pH adjusted. Ringer's lactate egg yolk (RLY) and coconut water egg yolk (CWY) were added with 9% chicken egg yolk and then centrifuged at 2000 rpm for 20. The supernatant was used as a basic extender with penicillin 1000 IU mL<sup>-1</sup> (PT. Meiji, Indonesia), 1 mg mL<sup>-1</sup> streptomycin (PT. Meiji, Indonesia) and 80 mM glucose (Merck, KgaA, Darmstadt Germany) were stirred for 5 minutes. The extender was adjusted to a pH of 6.5 using tris (hydroxymethyl) aminomethane (Merck, KgaA, Darmstadt Germany). Eight tubes were filled with different extenders. The first tube was filled with RLY + DMSO 7% (Merck, KgaA, Darmstadt Germany), the second tube was filled with CWY + DMSO 7%, the third tube was filled with RLY + EG 3% (Merck, KgaA, Darmstadt Germany), the fourth tube was filled with RLY + EG 5%, the fifth tube was filled with RLY + EG 7%, the sixth tube was filled with CWY + EG 3%, the seventh tube was filled with CWY + EG 5%, and the eighth tube was filled with CWY + EG 7%.

### Dilution, freezing, and thawing

The minimum concentration of sperm used in the subsequent freezing process was 2400 x 10<sup>6</sup> mL<sup>-1</sup>, and sperm motility was at least 80%. For the freezing process, the semen was divided into eight equal parts and placed into the tubes containing the extenders. The semen was divided into eight parts containing extenders RLY + DMSO 7%, CWY + DMSO 7%, RLY + EG 3%, RLY + EG 5%, RLY + EG 7%, CWY + EG 3%, CWY + EG 5% and CWY + EG 7%, where the ratio of extenders to semen was 10: 1. Diluted semen was packaged in 0.25 mL straws (IMV, France). Each straw had a minimum sperm concentration of 60 x 10<sup>6</sup> mL<sup>-1</sup>.

The straws were cooled to 5 °C for 2 hours for equilibration. The semen straws were frozen at 5 cm above liquid nitrogen for 10 minutes (Bearden et al. 2004) and stored in a liquid nitrogen container for 24 hours. The semen straws were thawed at 37°C for 30 seconds using a water bath (Shah et al. 2016), and then the sperm was evaluated. The recovery rate of motility was calculated by dividing the post-thaw motility by the sperm motility of fresh semen and presented as a percentage.

### Statistical analysis

Statistical analysis for multiple comparisons was performed using a completely randomized design with eight treatment levels and seven replications. Motility, viability, and abnormality after dilution and after freezing-thawing and recovery rate were analyzed using ANOVA, if the F-value is significant (P <0.05) then it was followed by Duncan multiple range test. Statistical analysis used SPSS 16 applications on Windows.

## RESULTS AND DISCUSSION

### Characteristics of fresh semen

The raw-semen characteristics of Indonesian native chicken were presented in Table 1. The average volume was 0.24 mL, thick consistency, milky white color, and a pH of 6.45. The average sperm mass activity was +++ which described thick and fast-moving sperm mass waves, sperm concentration of  $4054.17 \times 10^6 \text{ mL}^{-1}$ , sperm motility of 90%, sperm viability of 97.07%, and sperm abnormality of 7.73%. The characteristics of the semen had the standards to proceed to the freezing stage.

### Sperm quality after dilution

The result showed that there was no significant difference ( $P > 0.05$ ) in the motility and viability of sperm between treatments (Table 2). Sperm motility and viability were within the ranges of 87-88.43% and 96.55-98.25%, respectively. There was a significant ( $P < 0.05$ ) difference in sperm abnormality. The use of 7% DMSO resulted in a higher abnormality (8.24-8.67%) than the use of ethylene glycol (3.80-4.40%). However, no difference in abnormality was observed between 7% DMSO and ethylene glycol in coconut water egg yolk extender (5.88-6.34%).

### Spermatozoa quality after thawing

The results from the sperm motility, abnormality, and recovery rate showed that there was no significant difference ( $P > 0.05$ ) between the treatments (Table 3). Sperm motility was in the range of 24.28-32%, the abnormality was 6.74-9.05%, and the recovery rate was 27.1-35.74%. Sperm viability showed a difference ( $P < 0.01$ ) between treatments. The highest sperm viability was observed in the batch containing Ringer's lactate egg yolk extender with 7% DMSO (44.62%), while the other treatment showed low viability (24.64-33.78%).

**Table 1.** The average of fresh semen characteristics of Indonesia native chicken

Parameter	Mean $\pm$ SEM
Volume (mL)	0.24 $\pm$ 0.03
Color	Milk white
Consistency	Thick
pH	6.45 $\pm$ 0.10
Spermatozoa concentration ( $\times 10^6/\text{mL}$ )	4054.17 $\pm$ 610.2
Movement of spermatozoa mass	+++
Spermatozoa motility (%)	90.00 $\pm$ 1.75
Spermatozoa viability (%)	97.07 $\pm$ 0.42
Spermatozoa abnormalities (%)	7.73 $\pm$ 1.08

**Table 2.** Quality of Indonesian native chicken spermatozoa after dilution with LRY and CWY added with dimethyl sulfoxide and various levels of ethylene glycol

Treatment	Variable of spermatozoa (%) (mean $\pm$ SEM)		
	Motility	Viability	Abnormality
LRY + DMSO 7%	88.28 $\pm$ 0.61	96.76 $\pm$ 0.50	8.67 $\pm$ 1.21a
CWY + DMSO 7%	87.57 $\pm$ 0.68	97.67 $\pm$ 0.33	8.24 $\pm$ 1.04a
LRY + EG 3%	88.28 $\pm$ 0.61	97.13 $\pm$ 0.55	3.80 $\pm$ 0.90b
LRY + EG 5%	88.00 $\pm$ 0.75	97.09 $\pm$ 0.85	4.40 $\pm$ 0.76b
LRY + EG 7%	88.43 $\pm$ 0.78	96.82 $\pm$ 0.55	4.18 $\pm$ 0.73b
CWY + EG 3%	87.14 $\pm$ 0.55	98.25 $\pm$ 0.19	6.34 $\pm$ 0.46ab
CWY + EG 5%	87.43 $\pm$ 0.43	97.46 $\pm$ 0.70	5.88 $\pm$ 1.54ab
CWY + EG 7%	87.00 $\pm$ 0.95	96.55 $\pm$ 0.84	6.00 $\pm$ 1.36ab

Note: Different superscripts in the same column show significant differences ( $P < 0.05$ )

**Table 3.** Quality of Indonesian native chicken spermatozoa after thawing with LRY and CWY added with dimethyl sulfoxide and various levels of ethylene glycol

Treatments	Variable of spermatozoa (%) (mean $\pm$ SEM)			
	Motility	Viability	Abnormality	Recovery rate
LRY + DMSO 7%	32.00 $\pm$ 1.76	44.62 $\pm$ 2.40 <sup>a</sup>	8.91 $\pm$ 0.83	35.74 $\pm$ 2.00
CWY + DMSO 7%	31.14 $\pm$ 1.33	33.78 $\pm$ 3.16 <sup>b</sup>	8.83 $\pm$ 1.98	34.79 $\pm$ 1.54
LRY + EG 3%	27.71 $\pm$ 1.95	28.92 $\pm$ 3.81 <sup>b</sup>	8.41 $\pm$ 1.42	30.93 $\pm$ 2.13
LRY + EG 5%	26.86 $\pm$ 1.74	26.96 $\pm$ 3.37 <sup>b</sup>	9.05 $\pm$ 1.24	29.98 $\pm$ 1.91
LRY + EG 7%	27.28 $\pm$ 1.91	29.85 $\pm$ 2.69 <sup>b</sup>	7.40 $\pm$ 0.78	30.46 $\pm$ 2.09
CWY + EG 3%	27.28 $\pm$ 2.10	29.14 $\pm$ 3.88 <sup>b</sup>	7.06 $\pm$ 1.21	30.51 $\pm$ 2.42
CWY + EG 5%	26.14 $\pm$ 1.66	30.44 $\pm$ 3.08 <sup>b</sup>	6.74 $\pm$ 1.11	29.22 $\pm$ 1.93
CWY + EG 7%	24.28 $\pm$ 1.30	24.64 $\pm$ 2.44 <sup>b</sup>	6.83 $\pm$ 1.12	27.10 $\pm$ 1.41

Note: Different superscripts in the same column showed a very significant effect ( $P < 0.01$ )

## Discussion

The use of DMSO cryoprotectants and EG did not result in differences in motility after thawing, suggesting that both cryoprotectants have the same ability to maintain motility after thawing. This is consistent with the report of Mphaphathi et al. (2016) that no difference was found in total spermatozoa motility of Venda chickens with the use of DMSO and ethylene glycol for 1 hour and 2 hours of storage. The report also explained no differences in total motility, progressive motility, non-progressive motility, and sperm velocity traits following freezing-thawing of Venda cock semen supplemented with 8% DMSO and 8% ethylene glycol. However, the post-thaw sperm motility observed in this study was lower than the previous study that post-thawing chicken spermatozoa motility in Kovidil extender supplemented with DMSO was 46% and ethylene glycol 45% (Mphaphathi et al. 2016). This may be due to differences in the type of basic extender and the breed of chicken used.

The difference in sperm quality was determined through the viability of sperm after thawing. Ringer's lactate egg yolk extender containing DMSO maintained post-thaw spermatozoa viability better than extenders containing EG. This may be due to the low toxicity of DMSO, which resulted in higher sperm viability after the freeze-thaw process when compared to the low viability of sperm in extenders containing EG, which has higher toxicity levels. According to Bhattacharya (2018), ethylene glycol is metabolized into toxic elements when in warm conditions. Ethylene glycol has a high penetrating ability into the cell, which may cause damage to the cell membrane (Seshoka et al. 2016). Awad (2011) states that ethylene glycol has a lower molecular weight and a greater membrane permeability than DMSO. It is suspected that ethylene glycol permeates the sperm plasma membrane faster than DMSO, hence causing damage to the sperm during equilibration and cryopreservation (Gillmore et al. 2000). Sexton (1973) reported that ethylene glycol suppressed the respiration of chicken spermatozoa.

DMSO is low molecular weight and toxicity when used at low temperatures (Lake dan Ravie 1984). This is supported by Bhattacharya (2018) explaining that DMSO has low cost and minor cytotoxicity, which makes it a more prominent candidate for cryopreservation. The most important step in freezing spermatozoa cells is removing water from the cell before intracellular freezing occurs. If this dehydration does not occur, large ice crystals will form in the cells, leading to cell damage. Conversely, if there is extreme dehydration, the cells will experience dryness and die as a result. As stated by Chaytor et al. (2012), cryopreservation of cells using slow-freezing results in dehydration of the cell in response to increased osmotic pressure as electrolytes are concentrated outside the cell during extracellular ice growth.

Excessive dehydration can be prevented using cryoprotectants. Cell-penetrating cryoprotectants, such as DMSO, readily cross the cell membrane and decrease the concentration of intracellular electrolytes while maintaining greater cell volumes (Chaytor et al. 2012). Bui et al. (2013) explained that DMSO freely permeates cell

membranes due to its low hydrophilicity and molecular weight and is therefore thought to disrupt ice crystal nucleation and formation by forming hydrogen bonds with water. DMSO is a polar aprotic solvent that can dissolve polar and nonpolar compounds and can be easily miscible with a wide range of organic solvents and with water (Bhattacharya 2018). Cryoprotectants, simply by increasing the total concentration of all solutes in the system, reduce the amount of ice formed at any given temperature (Pegg 2015).

The results of this viability study are consistent with the report by Gerzilov (2010) that the mobility of post-thawing muscovy spermatozoa was lower by using ethylene glycol at 3%, 5%, and 7% when compared to use 7% DMSO. Sexton (1973) reported that sperm motility in 4% ethylene glycol was a reduction when compared to 4% glycerol or DMSO. Woelders et al. (2006) reported the use of EG as a cryoprotectant during chicken semen cryopreservation, where it was found to yield lower post-thaw motility and viability.

The viability of spermatozoa after thawing, if observed based on the type of based extender with the same cryoprotectant, namely DMSO, demonstrates that Ringer's lactate egg yolk extender maintains the viability of spermatozoa better than the coconut water egg yolk extender. This may be due to the presence of lactate in Ringer's solution, which is beneficial for spermatozoa. The research of Yamashiro et al. (2010) on rat spermatozoa showed that exogenous lactate in the freezing extender is a potent inducer that enhances the oxygen consumption of sperm and their motility after collection and freezing-thawing. Lactate is used by sperm as an essential substrate to maintain highly regulated ATP production and dissipation. The sperm can use exogenous lactate in the cryodiluent as an essential substrate to maintain highly regulated metabolic capacity, and that this lactate acts as an energy substrate for mitochondria to the mobilization of fresh and frozen-thawed sperm.

Ringer's lactate contains physiologic concentrations of sodium, chloride, potassium, calcium, and lactate (Albert et al. 2009). Ringer's lactate solution has potassium and calcium at concentrations that are similar to the ionized concentrations found in normal blood plasma (das Neves et al. 2019; Martini et al. 2013). According to Fujita et al. (2020), the ions in Ringer's solutions prevent cell death, and in maintaining cell viability. Rashid and Qistina (2015) reported the same as this study that Ringer's extender was the best compared with coconut water evaluated in both percentages of live sperm and abnormality of kampung cockerel throughout the 6-hour storage.

Sperm abnormality shortly after dilution was lower in the Ringer's lactate egg yolk extender containing EG compared to the extenders containing DMSO. According to Alcay et al. (2016), ethylene glycol successfully protected acrosomal and DNA integrities of ram spermatozoa. The sperm abnormality found in this study was quite low after dilution, and although there was a slight increase in abnormality after thawing, it remained below 10%. Bent head, midpiece, and tail were the main morphological abnormalities found in this study. According to Feyisa et

al. (2018), there was a difference in the proportion of spermatozoa with defective segments among breeds. The results of research by Ameen et al. (2014) stated that sperm abnormalities in the Hubbard and Isa White strains were dominated by head abnormalities. Most Korean native chicken sperm abnormalities were found to occur at the tail followed by the head (Feyisa et al. 2018). Viable abnormal sperm and defective tail were associated with poor motility, a high proportion of defective tail in an ejaculate volume will decrease motility, fertility, and hatchability as these defects might (Feysia et al. 2018).

In conclusion, dimethyl sulfoxide 7% was better than ethylene glycol 3%, 5%, and 7% in maintaining the quality of Indonesian native chicken frozen-thawed semen. While Ringer's lactate extender was better than coconut water when dimethyl sulfoxide added.

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