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Research Article
SK Kedu Semen Cryopreservation in Beltsville Poultry Semen Extender and Lactated Ringer's-Egg Yolk Extender Using Dimethyl Sulfoxide

[1]Sipora P. Telnoni, [2]R. Iis Arifiantini, [2]Tuty L. Yusuf and [3]Sri Darwati

[1]Reproductive Biology Programme, Post Graduate School, Bogor Agricultural University (IPB), 16680 Bogor, Indonesia
[2]Division of Veterinary Reproduction and Obstetrics, Department of Veterinary Clinic Reproduction and Pathology, Faculty of Veterinary Medicine, Bogor Agricultural University (IPB), 16680 Bogor, Indonesia
[3]Division of Animal Breeding and Genetics, Department of Animal Production and Technology, Faculty of Animal Science, Bogor Agricultural University (IPB), 16680 Bogor, Indonesia

Abstract

Objective: This study was conducted to evaluate frozen semen quality of SK kedu chicken after cryopreservation in Beltsville Poultry Semen Extender (BPSE) and lactated ringer's-egg yolk (LR-EY) with 8, 10 and 12% concentrations of dimethyl sulfoxide (DMSO). **Methodology:** Semen was collected from 5 SK kedu roosters using cloaca massage technique twice a week. Semen was evaluated macro and microscopycally then polled and divided into 6 tubes of treatments. Three tubes were diluted in $BPSED_8$, $BPSED_{10}$, $BPSED_{12}$ and three other tubes were diluted in $LR\text{-}EYD_8$, $LR\text{-}EYD_{10}$, $LR\text{-}EYD_{12}$. Semen of each treatments loaded into 0.25 mL straw, equilibrated at 5°C for 2 h. Freeze above nitrogen vapor and stored in container of liquid nitrogen at -196°C, then semen thawed in a water bath at 37°C for 30 sec. Data was analyzed using Statistical Analysis System (SAS). **Results:** Result showed that post thawing sperm motility in $LR\text{-}EYD_{10}$ (40.83±1.67%) was higher ($p<0.05$) than other treatments. The sperm recovery rate in $LR\text{-}EYD_{10}$ (46.71±1.97%) was higher than other treatments ($p<0.05$). Sperm viability was not differ post dilution, post equilibration and post thawing in all treatments. **Conclusion:** This study showed that LR-EY with 10% DMSO ($LR\text{-}EYD_{10}$) was better to maintain sperm motility and sperm recovery rate of SK kedu frozen semen.

Key words: Cryopreservation, SK kedu chicken, semen extender, bpse, lactated ringer's, egg yolk, sperm motility, sperm viability, sperm recovery rate, dimethyl sulfoxide

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Corresponding Author: R. Iis Arifiantini, Department of Veterinary Clinic Reproduction and Pathology, Faculty of Veterinary Medicine, Bogor Agricultural University (IPB), 16680 Bogor, Indonesia

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The SK kedu chicken is a cross-breeding within 3 local indigenous chickens from Indonesia i.e., sentul chicken, kampung chicken and kedu chicken. The SK kedu chicken provide benefits such as genetic resources diversity of poultry and one of the source protein in Indonesia. In efforts to support these benefits, conservation and increasing the population of SK kedu chicken are needed. Semen cryopreservation and Artificial Insemination (AI) are the reproductive biotechnology method that can be used to converse and increasing the population of SK kedu chicken. Semen cryopreservation is the most practical method for the long storage of poultry genetic resources[1] and AI is regarded as one of the assisted reproductive technique (ART's) to distribute the genetic material of livestock[2]. The implementation of chicken semen cryopreservation and AI for chicken still have a low successful. Sperm motility of chicken after thawing in Indonesia mostly ranging from 30.0-37.22%[3,4]. Furthermore, other research from outside by Blesbois[5] and Purdy[6] reported fertility rate after AI in hen were 30.4-60.0%. The reason of low successful in chicken semen cryopreservation because of the semen extender type, cryoprotectant type and concentration of cryoprotectant have not optimum to maintain sperm. Beltsville Poultry Semen Extender (BPSE) and Lactate Ringer's-Egg Yolk (LR-EY) are two semen extender in poultry. The BPSE has been used as semen extender with high fertiliy rate (88%) of white leghorn chicken[7]. The LR-EY extender on semen cryopreservation of Indonesian indigenous chickens mantain the sperm viability rate[4] at 48-49% and sperm viability rate at 69% on liquid semen preservation during 18 h at 4°C[8]. The DMSO is one cryoprotectant that has been used in cryopreservation of rooster semen[9]. The DMSO can maximize the replacement of water molecules in the cytoplasma, therefore formation of ice crystal is prevent and the cell membrane structure have a effective protection[10]. The addition of optimal concentration of cryoprotectant is assosiated with species. Gee *et al.*[11] reported 6% DMSO in American kestrel semen cryopreservation demonstrated 62% sperm motility after thawing, whereas in duck semen cryopreservation with 10% of DMSO showed 58% sperm motility after thawing[12]. This study aims to evaluate frozen semen quality of SK kedu chicken after cryopreservation in BPSE and LR-EY extender with different concentration of DMSO (8, 10 and 12%).

MATERIALS AND METHODS

Experimental animals and ethical approval: This study was conducted after approval by the Animal Care and Use Committee (ACUC) of Bogor Agricultural University with number: 028/ACUC/10/2016. The 5 SK kedu roosters age 48 weeks were used in this study, all roosters fed with 100 g commercial diet (17% crude protein) individual 1 day^{-1} and water was provided *ad libitum*. The roosters were housed in individual battery cages under tropical natural environmental conditions.

Extender preparation: All reagents were obtained from Merck, KgaA (Darmstadt Germany) unless otherwise indicated. Two extenders were use in this study, BPSE (Table 1) and lactated ringer's (LR) egg yolk. The LR and egg yolk mix well and was centrifuged at 2000 rpm for 15 min (Table 2). The supernatant was collected as extender. The BPSE and LR egg yolk were added with 8, 9 and 10% DMSO (v/v) (Table 3). All extenders were added with 1000 unit penicillin and 1 mg streptomycin mL^{-1} solution then stored at ambient temperature.

Semen collection and evalution: Semen of SK kedu was collected twice a week by cloaca massage technique early morning dan the semen transfered to the laboratory. Semen were evaluated macro and microscopycally. Semen evaluation conducted according to Arifiantini[13], with some modification. Macroscopically semen evaluation on volume, pH, color and consistency. Color and consistency were evaluated visually. Semen volume (mL) was measured using measuring pipette and potencial hydrogen (pH) using indicator paper (Merck scala 6.4-8). Microscopically semen evaluation for mass movement, sperm motility, sperm viability, sperm

Table 1: Chemical composisition of beltsville poultry semen extender (BPSE)

Constituents	g/100 mL
Potassium diphosphate (trihydrate)	1.270
Sodium glutamate (monohydrate)	0.867
Fructose	0.500
Sodium acetate (anhydrous)	0.430
Tris*	0.195
Potassium citrate (monohydrate)	0.064
Potassium monophosphate	0.065
Magnesium chloride	0.034
Aquabidest (mL)	100.00
pH	7.50
Osmotic pressure (mOsm kg^{-1} H_2o)	333.00

*Tris (hydroxymethyl)-aminomethan is used to replace TES (N-tris [hydroxymethyl] methyl-2-aminoethanesulfonic acid), Source: El-Gendy *et al.*[7]

Table 2: Composisition of lactated ringer's-egg yolk (LR-EY) extender

Constituent	mL
Lactated ringer's*	80.0
Egg yolk	20.0
pH	6.8
Total	100.0

*Lactated ringer's: Commercial solution (PT Emjebe Pharma)

Table 3: Extender composition for rooster semen cryopreservation

Constituent	$BPSED_8$	$BPSED_{10}$	$BPSED_{12}$	$LR\text{-}EYD_8$	$LR\text{-}EYD_{10}$	$LR\text{-}EYD_{12}$
BPSE (%)	92	90	88	-	-	-
LR-EY (%)	-	-	-	92	90	88
DMSO (%)	8	10	12	8	10	12
Penisilin (IU mL^{-1})	1000	1000	1000	1000	1000	1000
Streptomisin (mg mL^{-1})	1		1	1	1	1
Total (100%)	100	100	100	100	100	100

$BPSED_8$: BPSE+DMSO 8%, $BPSED_{10}$: BPSE+DMSO 10%, $BPSED_{12}$: BPSE+DMSO 12%, $LR\text{-}EYD_8$: LR-EY+DMSO 8%, $LR\text{-}EYD_{10}$: LR-EY+DMSO 10%, $LR\text{-}EYD_{12}$: LR-EY+DMSO 12%

Table 4: Sperm motiliy (%) of SK kedu semen on BPSE and LR-EY extender using 8, 10 and 12% concentration of DMSO

		Sperm motility (Mean±SEM) (%)		
Semen extender	DMSO (%)	Post dilution	Post equilibration	Post thawing
BPSE	8	63.48±1.57	62.03±1.04	34.58±1.36^{bc}
	10	63.77±0.75	60.33±1.69	34.17±1.06^{c}
	12	62.34±1.21	61.16±2.07	34.58±1.01^{bc}
LR-EY	8	63.52±1.21	59.77±1.13	38.75±1.25^{ab}
	10	63.86±1.41	62.10±1.49	40.83±1.67^{a}
	12	60.91±1.61	60.88±1.65	34.29±1.95^{c}

$^{a-c}$Values with different superscripts in the same columns are statistically different ($p<0.05$)

concentration and sperm morphology. Mass movement was evaluated by putting one drop of semen on the warm object glass and evaluated under light microscope at 100× magnification. Sperm motility was evaluated by putting one drop of semen on warm object glass and mix well with 4 drops of saline solution and covered with a cover glass and semen was evaluated under light microscope at 400× magnification. Sperm motility was subjectively assesed from 5 different fields at 400× magnification.

Sperm viability (%) and sperm morphology (%) were evaluated using eosine-nigrosin staining. One drop of semen and 5 drops of eosine-nigrosin putting in a clean object glass, homogenized, smeared and dried above heating plate. The smear then evaluated under light microscope at 400× magnification. Sperm concentrations (10^6 sperm cell mL^{-1}) were determined using Neubauer chamber.

Semen processing and freezing: Semen demonstrated >70% sperm motility, <20% abnormality and $>3000\times10^6$ sperm cells mL^{-1} in concentration used for this study. To avoid an individual variation, all semen were pooled and divided into 6 tubes. The three tubes were diluted in BPSE with DMSO 8% ($BPSED_8$), BPSE with DMSO 10% ($BPSED_{10}$), BPSE with DMSO 12% ($BPSED_{12}$) and other three tubes were diluted with LR-EY with DMSO 8% ($LR\text{-}EYD_8$), LR-EY with DMSO 10% ($LR\text{-}EYD_{10}$), LR-EY with DMSO 12% ($LR\text{-}EYD_{12}$). Diluted semen containing 400×10^6 sperm cells mL^{-1} (100×10^6 sperm cells $straw^{-1}$). The semen than packed into mini straw (0.25 mL) and labeled, all straws were placed at freezing rack and equilibrated on 5°C for 2 h. Immediately after equilibration, the straws freeze above liquid nitrogen vapor for 10 min then immersed into the liquid nitrogen container (-196°C) for further evaluation.

Frozen thawed semen evaluation: Straws of each treatments were thawed in a water bath at 37°C for 30 sec. Semen was evaluated for its sperm motility (%) and sperm viability (%). Sperm recovery rate (%) was measured by dividing frozen thawed sperm motility with sperm motility of fresh semen ×100%.

Data analysis: Data was analyzed using Statistical Analysis System (SAS) programme. Duncan's multiple range test has used as a tool to showed a significantly difference ($p<0.05$) between treatments. Data are presented as mean±standard error of mean (SEM) of measurements on treatments from 6 replicates.

RESULTS AND DISCUSSION

In this study, the macroscopically of SK kedu chicken semen quality were semen volume (0.15±0.02 mL), the color of semen (milky white), semen consistency (thick) and pH (6.93±0.06) and the microscopically were mass movement (3), sperm motility (82.75±1.38%), sperm viability (90.78±1.59%), sperm abnormality (2.44±0.63%) and sperm concentration ($4161\pm685.45\times10^6$ cell mL^{-1}). The semen quality included into this experiment were in the physiological range for chicken semen. In quality of frozen semen, sperm motility showed in 3 steps of semen cryopreservation are post dilution, post equilibration and post thawing (Table 4). Post dilution and post equilibration

Table 5: Sperm viability (%) of SK kedu semen on BPSE and LR-EY extender using 8, 10 and 12% concentration of DMSO

		Sperm viability (Mean±SEM)		
Semen extender	DMSO (%)	Post dilution	Post equilibration	Post thawing
BPSE	8	68.36±0.99	64.22±1.29	63.53±2.48
	10	69.88±0.77	64.36±1.78	59.66±0.75
	12	69.52±0.64	62.57±1.21	60.42±1.81
LR-EY	8	69.46±1.05	65.00±1.43	59.81±0.56
	10	69.98±1.55	66.64±0.85	58.09±1.35
	12	71.65±0.57	64.79±1.19	61.00±0.81

were not differ (p>0.05) on the sperm motility between treatments. This fact suggested that BPSE and LR-EY extender added with 8, 10 and 12% DMSO concentrations, maintain the progressive motility of sperm. Extenders have important roles as regulators of osmotic pressure, pH and ionic components[14]. The BPSE containing fructose as a source of energy to sperm and tris to maintain the pH and other chemicals subtantion including sodium glutamate as a chelator to protect against toxic ions, wherease LR-EY extender containing some important ionics on lactate ringer and egg yolk that contribute nutrients and energy for sperm. About 8, 10 and 12% of DMSO are in normal range concentrations and had no effect during equilibration. Sperm motility post thawing in LR-EYD_{10} (40.83±1.67%) was higher than other treatments. The LR-EYD_{10} had a good ability to protect sperm membrane plasma during freezing, storage and post thawing process. This ability was estimated by the compositions of LR-EY extender such as egg yolk which contain lecithin and phospholipids to maintain plasma membrane of sperm. Egg yolk is a common component of most semen cryopreservation extender that has been shown to have a beneficial effect on sperm cryopreservation to protect plasma membrane againts cold shock by lecithin and phospholipids actions[15-17]. Combination of 10% DMSO with LR-EY was estimated increase the membrane fluidity post thawing and had the highest ability to minimize osmolarity effect under cryopreservation condition. Rosato and laffaldano[18] explained that intacelluler cryoprotective agents are needed to increase membrane fluidity and to prevent dehydrating the cell. Similar optimum concentration 10% DMSO was reported by Rosato and laffaldano[18] in rabbit sperm cryopreservation which resulted the highest survival rate in post thawing motility and membrane integrity and other result by Blanco *et al.*[19] reported using 10% DMSO given the highest fertility rate (73.90%) in sandhill crane after insemination. The $BPSED_{10}$ (34.17±1.06%) and LR-EYD_{12} (34.29±1.95%) had the lowest progressive sperm motility. Spermatozoa in $BPSED_{10}$ was estimated had less protection. The BPSE had no component which protect sperm membrane during freezing. Spermatozoa in LR-EYD_{12} suggested undergo DMSO toxicity, 12% DMSO seems to be high concentration for rooster sperm. It is generaly accepted that the toxicity of a penetrating cryoprotective agents increase with its concentration[20] and during thawing, with temperature increasing, the toxicity of DMSO is increased and thus the cell damage is increased[10]. Decreased of sperm motility (%) at post thawing was estimated by cryoinjury of sperm cells when freezing for sperm cells were exposed to the very low temperature that made cold shock and cryoinjury of sperm cells when temperature changes during thawing. Cryoinjury is not only happened when freezing process but also occur during thawing[21].

Sperm viability (%) after cryopreservation is affected by the ability of semen extender to provide the nutrients and protect sperm cells from cold shock, whereas cryoprotectant prevent the formation of intracellular ice crystals that causes damage of sperm cells and injuring organelles during freezing and thawing procedur. Sperm viability (%) post dilution, post equilibration and post thawing are shown in Table 5.

Sperm viability (%) post dilution, post equilibration and post thawing between treatments were not differ (p>0.05). This fact showed that BPSE and LR-EY extender with 8, 10 and 12% DMSO maintain sperm cells integrity during all steps of cyopreservation procedur. A successful cryopreservation is effected by optimization of all steps on cryopreservation, including semen collection and dilution, equilibration with a suitable cryoprotectant, freezing in liquid nitrogen vapor, storage in liquid nitrogen and thawing before insemination[22]. Recovery rate is assosiated with the recovery capabilities of sperm after freezing[23]. Values of sperm recovery rate are shown in Table 6. Based on this research sperm recovery rate in LR-EYD_{10} (49.35±2.03%) was higher than other treatments, followed by LR-EY_8 (46.83±1.53%). The LR-EYD_{12} (41.45±2.36%) was the lowest value and followed by three treatments of $BPSED_8$, $BPSED_{10}$ and $BPSED_{12}$ (41.80±1.63, 41.80±1.21 and 41.30±1.27%). It was estimated that LR-EYD_{10} has the better ability to protect membrane integrity of sperm and provide energy for sperm movement. Motility recovery appears to be related to a more rapid and complete recovery of membrane integrity and permeability and perhaps to a be

Table 6: Sperm recovery rate of SK kedu semen in BPSE and LR-EY extender using 8, 10 and 12% concentration of DMSO

		Sperm motility (Mean±SEM)		
Semen extender	DMSO (%)	Fresh semen	Post thawing	Recovery rate
BPSE	8	82.75±1.38	34.58±1.36	41.80±1.63^{bc}
	10	82.75±1.38	34.17±1.06	41.30±1.27^{c}
	12	82.75±1.38	34.58±1.01	41.80±1.21^{bc}
LR-EY	8	82.75±1.38	38.75±1.25	46.83±1.53^{ab}
	10	82.75±1.38	40.83±1.67	49.35±2.03^{a}
	12	82.75±1.38	34.29±1.95	41.45±2.36^{c}

$^{a-c}$Values with different superscripts in the same columns are statistically different ($p<0.05$)

more efficient preservation of the biosynthesis and use of adenosine triphosphate (ATP) in the axonema[24]. Recovery rate can be used to estimated the ability of semen extender and concentration of cryoprotectant which maintain sperm cells to passing the critical steps during cryopreservation besides sperm motility and sperm viability.

CONCLUSION

Lactated ringer's-egg yolk (LR-EY) extender which added 10% DMSO (LR-EYD$_{10}$) was superior than other treatments to maintain sperm motility, post thawing and sperm recovery rate, whereas in all treatments have similar ability to maintain sperm viability post dilution until thawing.

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