Freezability and fertility of Thai native chicken semen in different diluents

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Abstract

The objective of the present study was to compare the efficacy of three diluents on post-thaw motility, viability, and fertility of frozen chicken semen. Semen from Thai native cocks (Pradu Hang Dam; n=24) was collected, pooled, and randomly allocated into three groups, then diluted at a ratio of 1:3 (v:v) with Schramm, EK, and polyvinylpyrrolidone (PVP)-based medium. The diluted semen samples were cooled down to 5°C and then mixed with Dimethylformamide (DMF) (6% v/v). Semen straws were subjected to cryopreservation using liquid nitrogen vapor method. For cryopreserved sperm quality assessment, the frozen semen straws were thawed, and motility was assessed by computer-assisted sperm analysis (CASA). Viability was evaluated using a SYBR-14/PI live/dead sperm viability kit. Lipid peroxidation level was measured based on malondialdehyde (MDA) levels. Fertility was also examined by inseminating layer hens (n=36) with the frozen semen. The experimental results showed that sperm motility and viability were superior when using Schramm and EK diluents, compared to PVP-based medium (P ≤ 0.05). The Schramm and EK diluents yielded lower levels of MDA and higher fertility than the PVP-based medium (P ≤ 0.05). The present study indicated that Schramm and EK diluents were the suitable cryopreservation media for native chicken; the diluents could maintain the quality of native chicken semen after freezing-thawing.

Keywords: fowl semen, extender, cryopreservation, fertility

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**Introduction**

Artificial insemination (AI) technology is important to commercial turkey meat production. This is due to the contrast in the size of the toms and hens, which results in low fertility rates after natural mating. AI is not commonly used in other poultry species (Donoghue and Wishart, 2000). However, AI may become essential in the future for commercial chickens (broiler breeds), intensifying genetic selection for rapid growth and increased breast size which are restricted by natural mating (Ramachandran, 2014).

The importance of AI in poultry reproduction both for commercial production and genetic conservation has stimulated research interest in developing the proper diluents and techniques for liquid and frozen semen storage (Donoghue and Wishart, 2000; Blesbois, 2007). The challenges are due in part to some special physiological properties of avian semen which make it susceptible to damage during the freeze-thaw process (Cerolini et al., 1997), leading to membrane rupture, and thus affecting semen quality and fertilizing ability (Long, 2006). Therefore, a suitable freezing medium is crucial for post-thaw spermatozoa fertilizing ability.

EK was reported to be an effective diluent for storing fowl semen (Łukaszewicz, 2002) and PVP-based medium was found to protect rooster sperm cells against cold shock and during freezing and thawing (Santiago-Moreno et al., 2012). In our laboratory, Schramm diluent is normally used for frozen chicken semen processing. However, very little is known about diluents appropriate for freezing chicken semen using the simple vapor method, since specific diluents may require an optimal freezing protocol.

The objective of the present study was, therefore, to compare the effects of different diluents on the quality and fertility of cryopreserved Thai native chicken semen under conditions of simple vapor freezing method, a practical protocol for semen cryopreservation under field conditions.

**Materials and Methods**

**Chicken Raising and Experimental Design:** Twenty-four Thai native cocks (Pradu Hang Dam), ten months of age, were used in this study. The animals were kept in individual cages. Cockerels were fed 130 g/head/day, and water was provided ad libitum. For fertility testing, 48 females (40 weeks of age and mean laying rate of 85%) were used. The animals were reared under natural environmental conditions throughout the experiment. The study was conducted at the research farm of the Department of Animal Science, Faculty of Agriculture, Khon Kaen University, Thailand, under approval of the Animal Ethics Committee (Approval No: 0514.1.12.2/31) of Khon Kaen University.

Semen from each individual was collected by the dorso-abdominal massage method (Burrows and Quinn, 1937). The semen samples were evaluated under a microscope and were selected on the basis of meeting the following criteria: mass motility score ≥ 4 (score range 0-5), sperm concentration ≥ 3 x 10^9 sperm/mL (hemocytometer counting method), and sperm viability ≥ 90% (nigrosin-eosin staining).

**Dilution and Cryopreservation Procedure:** Pooled semen samples were divided into three aliquots and diluted to 1:3 (v/v) with Schramm, EK (modified IGK, by Łukaszewicz, 2000), and polyvinylpyrrolidone (PVP)-based medium (Table 1). The diluted semen samples were cooled down from 25°C to 5°C for 1 h, and N,N-Dimethylformamide (DMF) (Sigma Aldrich, St.Louis, MO, USA) was added to a final concentration of 6% (v/v). The semen was immediately loaded into 0.5 mL plastic straws (IMV Technologies, L’Aigle, France), sealed with polyvinylpyrrolidone (PVP) powder (IMV Technologies, L’Aigle, France), and equilibrated at 5°C for 15 min. After equilibration, the filled straws were laid horizontally on a rack 11 cm above the surface of liquid nitrogen (-135°C) for 12 min, then placed 3 cm above liquid nitrogen vapor (-135°C) for 5 min, and subsequently immersed in liquid nitrogen (Vongpralub et al., 2011). The semen straws were transferred to a liquid nitrogen container for storage. After storage for at least 3 days, the straws were thawed individually in an ice water bath at 5°C for 5 min and then evaluated for various sperm functions.

**Table 1**  Poultry semen extenders

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Schramm</th>
<th>EK</th>
<th>PVP-based medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magnesium acetate</td>
<td>0.07</td>
<td>0.14</td>
<td>0.08</td>
</tr>
<tr>
<td>Potassium citrate</td>
<td></td>
<td>0.14</td>
<td></td>
</tr>
<tr>
<td>Potassium acetate</td>
<td>0.50</td>
<td>1.40</td>
<td>1.92</td>
</tr>
<tr>
<td>Sodium glutamate</td>
<td>2.85</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium dihydrogen phosphate</td>
<td>0.21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protamine sulfate</td>
<td>0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anhydrous sodium hydrogen phosphate</td>
<td></td>
<td>0.98</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>0.50</td>
<td>0.70</td>
<td></td>
</tr>
<tr>
<td>Fructose</td>
<td></td>
<td>0.20</td>
<td></td>
</tr>
<tr>
<td>Inositol</td>
<td>0.25</td>
<td>0.70</td>
<td></td>
</tr>
<tr>
<td>Polyvinylpyrrolidone</td>
<td></td>
<td>0.10</td>
<td>0.30</td>
</tr>
<tr>
<td>Osmolality (mOsm)</td>
<td>395</td>
<td>390</td>
<td>360</td>
</tr>
</tbody>
</table>

Adapted from Schramm (Schramm, 1982), EK (Łukaszewicz, 2000), PVP-based medium (Santiago-Moreno, 2012)
Assessment of Frozen/Thawed Sperm Quality: For each assessment of motility, the frozen sperm was diluted in each extender at a ratio of 1:15 according to the motility analysis procedure suggestion by IVOS clinical guide, dropped onto 2X-CEL slide sperm analysis chamber and covered with coverslip. Sperm motility was evaluated using computer-assisted semen analysis (CASA).

Sperm membrane integrity was assessed with dual fluorescent probes, SYBR-14 and Propidium Iodide (PI) (LIVE/DEAD® Sperm Viability Kit; Invitrogen™, Thermo Fisher Scientific, Waltham, MA, USA) according to the method described by Partyka et al. (2012). PI-negative and SYBR-14-positive population showing green fluorescence was considered to be live, with sperm plasma membrane intact (PMI).

Lipid Peroxidation: Assessment was done by analyzing the amount of malondialdehyde (MDA) formed by thiobarbituric acid reactive species (TBARS). The MDA procedure was similar to that described by Aisen et al. (2005).

Fertilizing Capacity: For fertility test, the straws were thawed in an ice water bath at 5°C for 5 min. All hens (12 hens/treatment) were inseminated once with the frozen/thawed semen (500×10⁶ sp2) from each group at a dose of 0.4 mL. Artificial insemination was performed between 3:00-5:00 pm. Eggs were collected on days 2-8 after each insemination. Fertility was determined by candling eggs on day 7 of incubation. Six replications of the fertility test were carried out. The hens were artificially inseminated once every 14 days.

Statistical Analyses: The experiment was conducted as a randomized complete block design (RCBD). Results are presented as mean ± SE. Means were analyzed with ANOVA followed by Duncan’s multiple range test to determine significant differences in all the parameters between groups. All percentage data were arcsine transformed before statistical analysis. A probability level of P ≤ 0.05 was considered as significant. Six replications were conducted for all of the parameters.

Results and Discussion

Determination of the proper diluent is critical from the point of view of semen cryopreservation. The present study was undertaken to compare suitable diluents for cryopreservation of native chicken semen under conditions of simple vapor method and using DMF as a cryoprotective agent. Results were interpreted in terms of post-thaw quality and fertility of chicken semen. Results of the effects of different extenders on post-thaw quality of cryopreserved chicken semen are presented in Table 2. The study found that the Schramm diluent yielded a higher viability rate of sperm compared with the PVP-based medium; however, there was no significant difference (P > 0.05) when compared with the EK diluent. The motility characteristics as analyzed by CASA showed that the percentage of total motile sperm (MOT) with the Schramm and EK diluents revealed a higher motility rate than with the PVP-based medium. However, progressive motile sperm (PMOT) was not different among the treatments (P > 0.05). According to our former studies using Schramm as the freezing diluent, results of percentage of motility (46.33% and 47.00%) and percentage of progressive motile (29.16% and 27.25%) were similar to the present study (Thananurak et al., 2015; Thananurak et al., 2016). Other previous reports showed that using PVP-based medium for semen cryopreservation resulted in low percentage of sperm motility (9.7%), progressive motile (3.0%) and viability (13.3%) (Santiago-Moreno et al., 2012). Partyka et al. (2012) reported that EK extender gave post-thaw sperm viability of 37.3%. Sittikasamkit et al. (2015) reported that Schramm diluent improved live with intact acrosome (37.43%) and live spermatozoa with functional mitochondria (46.25%). The results of this study revealed that post-thaw sperm viability and motility were also affected by semen diluent. Chicken sperm can survive in extenders with osmolality from 250 to 460 mOsmol/kg (milliosmoses per kg of water) (Christensen, 1995). However, the ideal osmotic pressure, as recommended by Sexton (1973), is 325 to 350 mOsmol/kg. Interestingly, Sakhatky (1990) stated that the specific constituents of diluents were less important than the overall osmotic pressure, which should be hypertonic compared with that of the animal plasma of each species by 50 to 100 mOsmol/kg. As reported by Surai and Wishart (1996), chicken semen osmolality was 310 to 338 mOsmol/kg and plasma osmolality of fowl was approximately 320 mOsmol/kg (Mongin, 1976). In our preliminary study, the osmolality of Thai native chicken semen was found to be 305 to 338 mOsmol/kg. Based on osmotic pressure considerations, the PVP-based diluent may be the least effective freezing extender and the high osmolality of Schramm and EK could affect osmotic pressure. Sugars are normally a component of semen diluents and have several functions in the extender, not only serving as an energy source for maintaining osmotic pressure, but also acting as a cryoprotectant during the cryopreservation process (Pursel, 1979). Sperm requires energy for survival and movement; hence, diluents may be supplemented with energy substrates in order to prolong viability and activity (Christensen, 1995). In this study, monosaccharide was added to the Schramm and EK diluents, whereas the PVP-based medium contained no sugar. Based on the energy source considerations suggesting that sugars have a positive effect, the Schramm and EK diluents were found to be superior extenders for semen cryopreservation compared with the PVP-based medium.

In terms of the quantity of MDA, an end product of the lipid peroxidation reaction, the amounts of MDA measured for the Schramm and EK diluents were lower than for the PVP-based medium (Table 3). The results from fertility testing are summarized in Table 3. The Schramm and EK diluents yielded the best fertility of frozen semen compared with the PVP-based medium (P ≥ 0.05). It is generally recognized that the utero-vaginal junction and the lower portion of the infundibulum are the main sperm storage sites) SSTs (in poultry. Sperm that reaches SSTs must be motile and maintain an intact plasma
membrane (Blesbois and Brillard, 2007). In poultry, sperm quality tests highly significantly correlate with fertilizing ability (Wishart, 2009), suggesting that Schramm and EK were beneficial to post-thaw sperm survival in the fowl. Three interaction factors, the cryoprotectant, the rate of cooling, and the rate of thawing, are influenced by the semen freezing protocol (Hammerstedt, 1995).

In this study, the different effects of the diluents on post-thaw semen quality and fertility could be due to the suitability of the diluent and the interaction of each diluent with the specific freezing protocol used, i.e., the simple vapor method. It has been suggested that the wide differences in poultry sperm freezability among lines and strains are linked to heritably physiological or biochemical, or both, attributes (Long et al., 2010). Schramm diluent and the simple vapor freezing method seemed to improve the post-thaw fertility of Thai native chicken semen (Thananurak et al., 2016). In our previous study, Schramm diluent not only yielded high fertility rate in Thai native chicken (Pradu Hang Dam), but also in Rhode Island Red post-thaw semen fertility. This may suggest that Schramm diluent may be suitable for cryopreservation of other cockerel lines apart from Pradu Hang Dam. According to the previous report of Siędzińska and Lukaszewicz (2006), EK was found to be the most suitable regardless of fowl breed for cool storage of semen. This diluent was also used in semen cryopreservation of gander (Łukaszewicz, 2001). In the present study, high fertility rate was obtained following AI with EK. Thus, EK may be one of the suitable diluents for semen cryopreservation in chicken.

Table 2 Percentage of sperm viability, motile sperm, progressive motile sperm, average path velocity, straight-line velocity, and curvilinear velocity of frozen/thawed sperm in Schramm, EK, and PVP-based medium*

<table>
<thead>
<tr>
<th>Item</th>
<th>Schramm</th>
<th>EK</th>
<th>PVP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viability (%)</td>
<td>49.9±0.21</td>
<td>48.75±0.57</td>
<td>43.97±0.90</td>
</tr>
<tr>
<td>MOT (%)</td>
<td>64.3±1.16</td>
<td>63.67±1.44</td>
<td>51.17±1.15</td>
</tr>
<tr>
<td>PMOT (%)</td>
<td>28.0±0.10</td>
<td>28.5±1.46</td>
<td>27.0±1.58</td>
</tr>
<tr>
<td>VAP (µm/s)</td>
<td>89.4±2.40</td>
<td>104.27±2.40</td>
<td>80.8±1.27</td>
</tr>
<tr>
<td>VSL (µm/s)</td>
<td>76.8±1.29</td>
<td>94.7±2.59</td>
<td>62.6±1.76</td>
</tr>
<tr>
<td>VCL (µm/s)</td>
<td>129.18±1.66</td>
<td>130.12±1.59</td>
<td>105.2±1.31</td>
</tr>
</tbody>
</table>

* superscript letters within columns indicate significant differences (P ≤ 0.05).

MOT = motile sperm, PMOT =progressive motile sperm, VAP =average path velocity, VSL =straight-line velocity, VCL =curvilinear velocity.

Table 3 Malondialdehyde concentration, based on level of lipid peroxidation, and fertility test in frozen/thawed chicken semen. Each sperm aliquot was diluted in Schramm, EK, and PVP-based medium*

<table>
<thead>
<tr>
<th>Semen sample and female group</th>
<th>MDA (µM·mL⁻¹·150·10⁸spz)</th>
<th>Number of set eggs</th>
<th>Fertility (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A - Diluted with Schramm</td>
<td>1.5±0.95</td>
<td>321</td>
<td>91.2±2.20</td>
</tr>
<tr>
<td>B - Diluted with EK</td>
<td>1.47±1.23</td>
<td>320</td>
<td>85.4±1.35</td>
</tr>
<tr>
<td>C - Diluted with PVP</td>
<td>2.80±0.86</td>
<td>315</td>
<td>66.7±2.34</td>
</tr>
</tbody>
</table>

* superscript letters within columns indicate significant differences (P ≤ 0.05).

MDA = Malondialdehyde

**Conclusion**

It can be concluded that Schramm and EK diluents are suitable cryopreservation media for native chicken because both could maintain the quality of native chicken semen after freezing-thawing under conditions of the simple vapor freezing method, a practical protocol for semen cryopreservation under field conditions. For economical reason, it is proposed that Schramm may be the most suitable diluent. The present research might be applied in the future for the preservation of sperm of various endangered native chickens in developing countries.

**Acknowledgements**

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**References**


บทคัดย่อ

คุณภาพของน้ำเชื้อไก่พื้นเมืองไทยที่เก็บรักษาด้วยวิธีแช่แข็งในน้ำยาเจือจางในน้ำเชื้อที่แตกต่างกัน

พัชรา ธนานุรักษ์ แอนภิกา ช่วยชูหนู เทวินทร์ วงษ์พระลับ

การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อเปรียบเทียบประสิทธิภาพของน้ำยาเจือจาง 3 สูตรในการรักษาคุณภาพของน้ำเชื้อไก่พื้นเมืองไทยที่เก็บรักษาด้วยวิธีแช่แข็งในน้ำยาเจือจาง 3 สูตร ได้แก่ Schramm EK และ PVP-based medium ในอัตราส่วน 1:3 (น้ำเชื้อ : น้ำยา) ท้าการลดอุณหภูมิไปที่ 5 องศาเซลเซียส จากนั้นเติม DMF (6% v/v) บรรจุน้ำแข็งในหลอดฟาง และทำการแช่แข็งโดยการควบคุมอุณหภูมิโดยเครื่อง CASA ประเมินอัตราการเคลื่อนที่โดยการย้อมฟลูออเรสเซนต์ชนิด SYBR-14 และ PI ประเมินอัตราการเกิดลิปอิกซิเดชันโดยวิเคราะห์จากความเข้มข้นของปริมาณ MDA และทดสอบอัตราการผสมติดของน้ำเชื้อแบบแช่แข็งโดยการผสมติดในแม่ไก่ไข่พันธุ์ทางการค้าท้าการประเมินอัตราการเคลื่อนที่ อัตราการรอดชีวิต อัตราการเกิดลิปอิกซิเดชันและอัตราการผสมติด ที่สูงสุดของสูตร PVP-based medium (P ≤ 0.05) อีกทั้งยังให้อัตราการเกิดลิปอิกซิเดชันและอัตราการผสมติดสูงกว่าสูตร PVP-based medium (P ≤ 0.05) การศึกษาครั้งนี้ชี้ให้เห็นว่า น้ำยาเจือจางสูตร Schramm และ EK ให้ผลดีในการเก็บรักษาชีวภาพแช่แข็งในน้ำเชื้อไก่พื้นเมืองไทย

คำสำคัญ: น้ำเชื้อไก่ น้ำยาเจือจาง การเก็บรักษาด้วยวิธีแช่แข็ง อัตราการผสมติด

1 ภาควิชาสัตวศาสตร์ คณะเกษตรศาสตร์ มหาวิทยาลัยขอนแก่น ขอนแก่น 40002
2 ศูนย์เครือข่ายวิจัยและพัฒนาด้านการปรับปรุงพันธุ์สัตว์ (ไก่พื้นเมือง) คณะเกษตรศาสตร์ มหาวิทยาลัยขอนแก่น ขอนแก่น 40002
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