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The Goat Semen Quality after Being Frozen Using Albumin and Cholesterol Substituted for Egg Yolk in Semen Extender

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Abstract

The objective of this study was to determine the effect of albumin and cholesterol substituted for egg yolk in goat semen extender after frozen-thawed on semen quality and pregnancy rate. The semen was collected from three bucks by using artificial vagina. The semen was divided into 5 groups; in group 1, seminal plasma was removed and 20% egg yolk extender was added (standard), in group 2, 2% egg yolk extender was added into whole semen (control), in group 3, 4 mg/mL albumin extender was added into whole semen, in group 4, 5 mg/mL cholesterol extender was added into whole semen, and in group 5, 4 mg/mL albumin and 5 mg/mL cholesterol extenders were added into whole semen. Semen was loaded into 0.25 mL straw and frozen by automatic semen freezing machine and stored in liquid nitrogen. The frozen semen was thawed at 37 °C for 30 seconds. It was found that the cholesterol-added group was not different from the standard control group but the albumin or the albumin-cholesterol-added groups were significantly lower ($P<0.05$) than both control groups when compared with microscopic semen quality, computer analyzed semen quality. The result of pregnancy rate by artificial insemination with frozen semen was not significantly different but tended to be lower in the albumin-added group. In conclusion, cholesterol-added extender substituted egg yolk in whole goat semen prove to be of practical use.

Keywords: frozen semen, goat, albumin, cholesterol, egg yolk

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Introduction

Semen extender is necessary for semen cryopreservation to increase volume, nutritional, and cryoprotection. The ability to adjust pH of semen from extender and cryoprotection are different between species. Appropriate pH of goat and sheep semen extender is 6.8-7.0 (Barbas and Mascarenhas, 2009). Egg yolk is used in semen extender before freezing for sperm membrane protection from the rapid decrease temperature in the freezing process of semen cryopreservation. For goat semen freezing, the egg yolk will have effects on frozen-thawed sperm quality because phospholipases enzyme from goat bulbourethral gland will have a reaction to lecithin in egg yolk. Lysolecithin from this reaction can be toxic to goat sperm resulting in loss of membrane integrity and loss of motility (Ritar and Salamon, 1982). In general, goat semen cryopreservation with 20-25% egg yolk in tris-citrate-glucose extender should be centrifuged to remove seminal plasma and reduce toxicity in the sperm (Ahmad and Foote, 1984; Ritar and Salamon, 1982). But Centrifugation of goat semen before freezing is time-consuming, results in sperm loss and the seminal plasma that contains beneficial factors to sperm (Pickett et al., 1975; Ritar, 1993; Konyali et al., 2013). The less than 2% of egg yolk should be used in semen extender but some toxic from egg yolk will have an effect on sperm (Ritar and Salamon, 1982).

The semen cryopreservation will damage sperm by cooling down the temperature from room temperature to 5°C. It can decrease the motilities, respiratory activities and glycolysis process of spermatozoa (Pickett and Komarek, 1967). This phenomenon is called cold shock and induces the change of sperm plasma membrane since membrane proteins and phospholipids will be separated and plasma membrane will lose the membrane integrity and membrane fluidity. Towards the end of semen cryopreservation process, sperm changed the intracellular chemicals, it is call cryocapacitation. The loss of cholesterol in the sperm plasma membrane from cryocapacitation can have an effect on sperm motility, and fertilization to the oocytes (Parks and Graham, 1992). The study of Berger et al. (1994) found that cholesterol added in semen extender substituted the cholesterol loss from sperm plasma membrane in the freezing process effect on the sperm tolerance to cryoinjury. The cholesterol couldn't be dissolved in water, but it can be loaded in cyclodextrins and cholesterol can be inserted into the sperm plasma membrane. Cholesterol-loaded cyclodextrine (CLC) will increase the cholesterol in the sperm plasma membrane and sperm increases the efficiency to the freezing point and tolerance to the damage from cryopreservation (Purdy and Graham, 2004). The study of Fardin et al. (2010) found that 1.5 and 2.25 mg/mL CLC in egg yolk tris extender will increase the sperm motility rate and acrosome integrity rate in goat semen. Recently, it has been found that treating ram sperm with CLC increases sperm cryosurvival rate, osmotic tolerance and fertilization to increase conception rate (Moce et al., 2009).

Bovine serum albumin (BSA) in one of the available substitution protein for egg yolk, BSA is a

large protein molecule and is found in the female reproductive tract fluids (Foote et al., 1993). BSA in ram semen extender increases the sperm motility and viability rate of frozen thawed sperms (Fardin et al., 2010). Hideaki et al. (2006) reported that BSA on egg yolk tris extender induced increased the membrane integrity and protected the sperm from cold shock in freezing process of ram semen. Therefore, the objective of this study was to investigate the effect of CLC, BSA and combination in goat semen extender. The post-thawed semen qualities by CASA and pregnancy rate from frozen-thawed goat semen were evaluated.

Materials and Methods

Animals: This study was conducted at the Huai Hongkhrai Royal Development Study Center, Doi Saket, Chiangmai Thailand. Semen from three mature Saanen bucks (*Capra aegagrus hircus*) was used. Fifty mature does were used to evaluate the pregnancy rate from frozen semen. Approval for use of the experimental animals was obtained from the ethical committee of Faculty of Veterinary Medicine, Chiang Mai University before the start of study.

Preparation of cholesterol loading methyl- β -cyclodextrin (CLC): Cholesterol was loaded into the methyl- β -cyclodextrin and 1 g methyl- β -cyclodextrin was dissolved into 2 mL of methanol in a glass tube. In another glass tube, 200 mg of cholesterol was dissolved into 1 mL of chloroform. An aliquot of 0.45 mL of cholesterol solution was combined with methyl- β -cyclodextrin solution, stirred until the solution was clear, and then poured into a glass Petri dish and the solvents were removed by the air for 1 day. The 5 mg/mL of CLC (1.25 mg/ 200x10⁶ sperm) in the extender was used in the experiment (Purdy and Graham, 2004).

Semen collection, evaluation, freezing and thawing: Semen (one load per buck) was collected using an artificial vagina (AV) from three bucks. After collection, the semen was placed in a water bath at 37 °C, and then was evaluated; the volume more than 0.5 mL, concentration higher than 2.5x10⁹ spermatozoa/mL, motility rate higher than 70% and primary sperm abnormality lower than 10% were used in this experiment. The individual semen was divided into 5 aliquots with tris-citrate-glucose (TCG) and 4% glycerol (v/v) extender to final concentration of 800x10⁶ spermatozoa/mL. The first aliquot was standard control, seminal plasma was removed from the semen by centrifugation, mixed with TCG and 20% egg yolk was used in extender (20TCG), the second aliquot (whole semen) was mixed with TCG and 2% egg yolk (2TCG), the third aliquot (whole semen) was mixed with TCG and CLC (TCG+CLC), the fourth aliquot (whole semen) was mixed with TCG and albumin (TCGB) and the fifth aliquot (whole semen) was mixed with TCG, albumin and CLC (TCGB+CLC) in table 1. Then, the diluted sperms were loaded into 0.25 mL straw, and frozen with automatic freezing machine (Freeze Control Cryology, Australia). The temperature decreased from 20 °C to -120 °C for 2 hours, and then plunged into the liquid nitrogen for

storage. The straws from each treatment were thawed in a water bath at 37 °C for 30 s before analysis.

Motion and kinetics characteristics: Post-thawed sperm total motility and progressive motility were evaluated using the computer-assisted semen analysis (CASA, Hamilton Thorn Motility Analyzer, IVOS 12.3, USA). The other motion parameters and sperm kinetics were evaluated as Distance curve line (DCL), Distance average path (DAP), Distance straight line (DSL), Velocity curve line (VCL), Velocity average path (VAP), Velocity straight line (VSL), Linearity (LIN) [VSL/VCL], Straightness (STR) [VAP/VCL], Beat cross frequency (BCF) and Amplitude of lateral head displacement (ALH).

Viability, membrane and acrosome integrity: The viability and normal morphology were measured by the eosin-nigrosin stain technique. After staining, slides were microscopically examined (400x) and spermatozoa were classified into dead (red head) and alive and normal and abnormal spermatozoa. The membrane integrity was measured by the hypo-osmotic swelling test by water test technique at 37°C for 30 min. Two hundred spermatozoa were classified into plasma membrane swelling or not. The Acrosome integrity was measured by the double stain technique. The sperm smear slides were stained with Congo red for 20 s, followed by crystal violet 10 s, washed, and evaluated under microscope (1000x). Two hundred sperms were classified as intact acrosome, partially damage acrosome and detached acrosome.

Table 1 Composition of experimental extenders used for buck semen including TCG, egg yolk, albumin and CLC.

Extenders	Centrifugation	Egg yolk (v/v)	CLC (mg/mL)	BSA (mg/mL)
20TCG (standard control)	500 G, 10 min	20%	-	-
2TCG (control)	-	2%	-	-
TCGB	-	-	-	4.0
TCG+CLC	-	-	5.0	-
TCGB+CLC	-	-	5.0	4.0

Table 2 Effect of CLC and BSA in semen extender on motion parameters and kinetic parameters of sperms in buck's semen after freezing-thawing.

Treatment	TMo (%)	PMo (%)	STR (%)	LIN (%)	VAP (micron/s)	VSL (micron/s)	VCL (micron/s)	ALH (micron/s)	BCF (Herz)
20TCG	23.9±3.3 ^a	12.2±1.4 ^a	76.6±2.2	45.4±1.6	69.7±2.5 ^a	53.7±1.8 ^a	125.8±4.7 ^a	6.2±0.3 ^{a,b}	26.9±1.0
2TCG	5.3±2.3 ^b	1.8±0.7 ^b	71.6±9.2	44.1±5.8	49.7±6.3 ^b	41.1±5.4 ^{a,b}	84.1±10.7 ^b	4.6±0.6 ^b	21.4±3.3
TCGB	2.2±2.0 ^c	0.9±0.4 ^b	75.6±3.5	47.0±2.5	53.2±3.2 ^b	42.4±4.2 ^{a,b}	90.7±5.1 ^b	4.9±0.7 ^b	26.3±2.2
TCG+CLC	15.2±2.9 ^a	8.7±1.9 ^a	71.7±2.0	39.8±1.5	73.3±1.5 ^a	53.3±1.1 ^a	143.1±5.4 ^a	7.0±0.2 ^a	28.7±0.5
TCGB+CLC	4.1±0.9 ^b	1.2±0.4 ^b	74.6±3.0	43.3±2.0	49.4±2.0 ^b	37.9±1.5 ^b	89.6±3.4 ^b	5.2±0.4 ^{a,b}	26.5±1.3

Different superscript letters indicate significant differences between means of the treatments at P<0.05. SEM indicates standard error of the mean.

Table 3 Effect of CLC and BSA in semen extender on sperm viability, normal sperm morphology and membrane integrity in buck's semen after freezing-thawing.

Treatment	Viability (%)	Normal sperm morphology (%)	Membrane integrity (%)
20TCG	52.4±0.9 ^a	90.4±0.5 ^a	49.9±0.8 ^a
2TCG	38.4±1.2 ^b	88.5±0.4 ^b	43.3±1.0 ^b
TCGB	29.9±2.0 ^c	89.9±0.4 ^{a,b}	31.8±1.0 ^c
TCG+CLC	57.2±1.1 ^a	90.6±0.5 ^a	50.8±1.0 ^a
TCGB+CLC	41.9±1.3 ^b	89.1±0.3 ^{a,b}	37.0±1.0 ^d

Different superscript letters indicate significant differences between means of the treatments at P<0.05. SEM indicates standard error of the mean.

Pregnancy diagnosis: The fifty does were divided into five groups. They were synchronized estrus using controlled internal drug release device for goat (CIDR-G[®]) inserted into vagina for 5 days. And then they were injected with 100 IU gonadotropin hormone (Folligon[®], Canada) and inseminated with 200x10⁶ frozen-thawed semen per straw for two times. Sixty days after transcervical artificial insemination, the pregnant does were evaluated with ultrasonography per rectal probe.

Statistical analysis: We designed, in this study, five treatments with ten replications. The results are presented as the least-squares means (LSM) ± the standard error of mean (SEM). Data of semen qualities were subjected to the variance analysis according to the one-way ANOVA and test the differences with Duncan Multiple Range Test (DMRT) using statistical package software (SPSS version 17, USA). Pregnancy rate of 60 day after insemination was statistically analyzed with

Chi-square test. A probability level of $P < 0.05$ was set to determine the different between treatment and control groups.

Results

Sperm motion and kinetic parameters: The effects of cholesterol and albumin on motion parameters (total motility, progressive motility) and kinetic parameters of goat sperm after frozen-thawed are presented in Table 2. The results revealed that extender containing TCG and CLC were, in regard to sperm motion parameters, significantly better than extender with 2% egg yolk, albumin or albumin and CLC ($P < 0.05$), but there was no significant difference from the standard control group (20% egg yolk). Additionally, extenders that contained CLC and 20% egg yolk revealed better sperm kinetic parameters such as STR, LIN, VAP, VSL, VCL, ALH than extender containing 2% egg yolk, albumin or albumin and CLC ($P < 0.05$) but with no significant differences in BCF parameter.

Viability, normal sperm morphology, membrane and acrosome integrity: The effects of CLC and albumin on viability, normal sperm morphology and membrane integrity of frozen-thawed goat semen are demonstrated in Table 3. The results showed that the extender containing CLC and 20% egg yolk were significantly better than extender with 2% egg yolk, albumin or albumin and CLC ($P < 0.05$). Moreover, Figure 1 displayed that there were not differences between treatment groups in acrosome integrity or intact acrosome but the control group (2% egg yolk) was significantly lower ($P < 0.05$).

Pregnancy rate: The effects of CLC and albumin on pregnancy rate of frozen-thawed goat semen are demonstrated in Figure 2. There were no significant differences between the treatments, standard and control group but tended to have higher pregnancy rate in the standard and extender with CLC when compared with others.

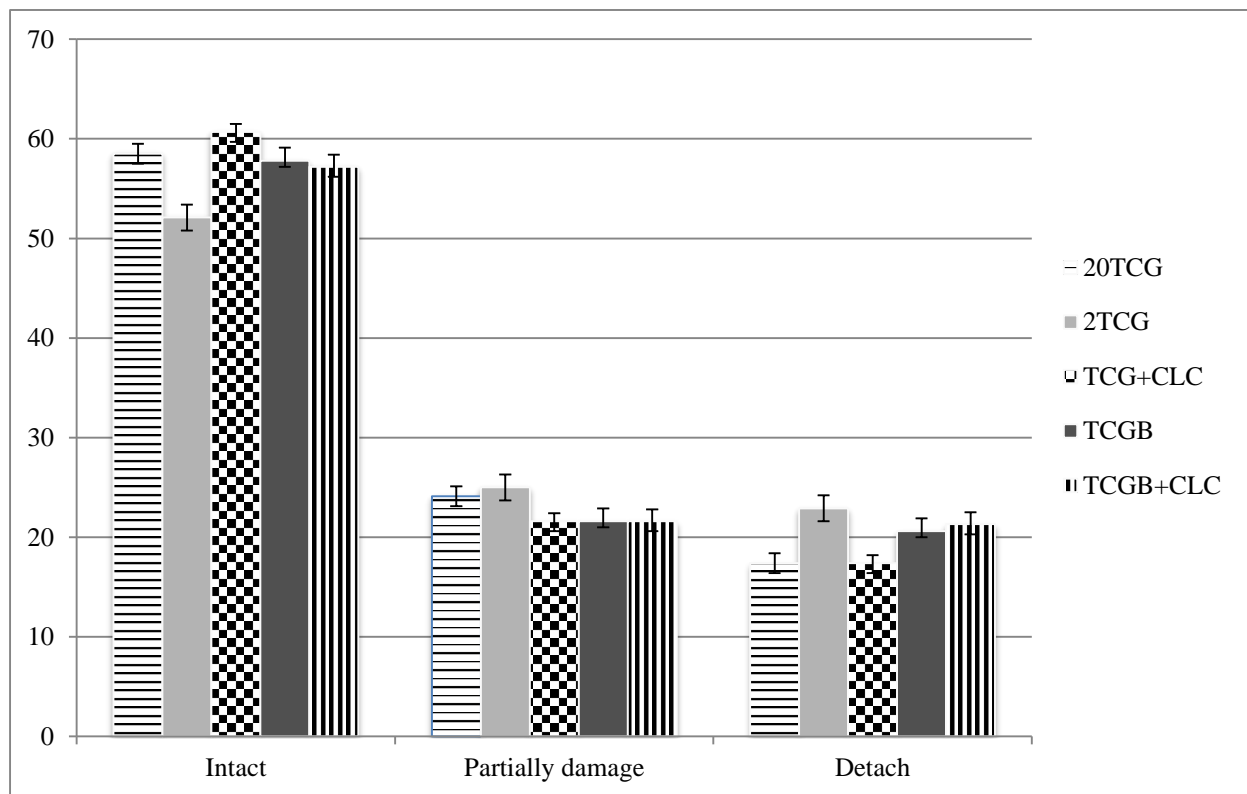


Figure 1 Effect of CLC and BSA on the mean percentages of acrosome integrity in buck's semen after freezing-thawing. Different letters indicate significant differences between means of the treatments at $p < 0.05$.

Discussion

The plasma membrane of sperm is the primary site of damage induced by cryopreservation resulting in reduced motility and fertility of spermatozoa (Parks and Graham, 1992). Membrane damage arises when plasma membrane undergoes a stage of transition from liquid crystalline to gel during cold shock and it was is typical of membrane to contain a high proportion of cholesterol depletion. On the other hand, sufficient levels of liposome containing cholesterol to sperm plasma membrane increased the cryo-tolerance (Wilhelm et al., 1996). As Purdy and

Graham (2004) puts it, the treatment of bull spermatozoa with CLC increased cholesterol in sperm plasma membrane and improved sperm resistance to cryoinjury due to cold shock and freezing damage.

In the present study, the effects of addition of CLC to TCG extender prior cryopreservation on buck sperm significantly increased of total motility, progressive motility, motion kinetics, viability and membrane integrity. Even though the mechanism by cholesterol enhances sperm quality after being thawed is not completely known, it is evident that cholesterol plays an integral role in controlling membrane fluidity. CLC treated sperms

presented higher cholesterol content in the membrane between 1.9-2.7 fold in bulls, stallions and rams (Purdy and Graham, 2004; Moore et al., 2005; Moce et al., 2009), which increases their resistance to cold shock, reduced membrane phase separations and therefore reduced leakage of cellular components or inhibit calcium entry into the sperm, which is a prerequisite for capacitation and senescence (Walters et al., 2008; Glazar et al., 2009). This could serve as a mechanism for the increase of frozen-thawed sperm survival rate, since osmotic

stresses during cryopreservation and thawing (Hammerstedt et al., 1990). In most cases, this CLC loading would therefore require to add the CLC to fixed amount of the sperm (1.5 mg CLC for 120×10^6 spermatozoa) (Amidi et al., 2010). In addition to it a higher dose of CLC (2 mg CLC for 120×10^6 spermatozoa) put an inhibitory effect on spermatozoa ability for capacitation and to undergo acrosome reaction (Aksoy et al., 2010; Serin et al., 2011)

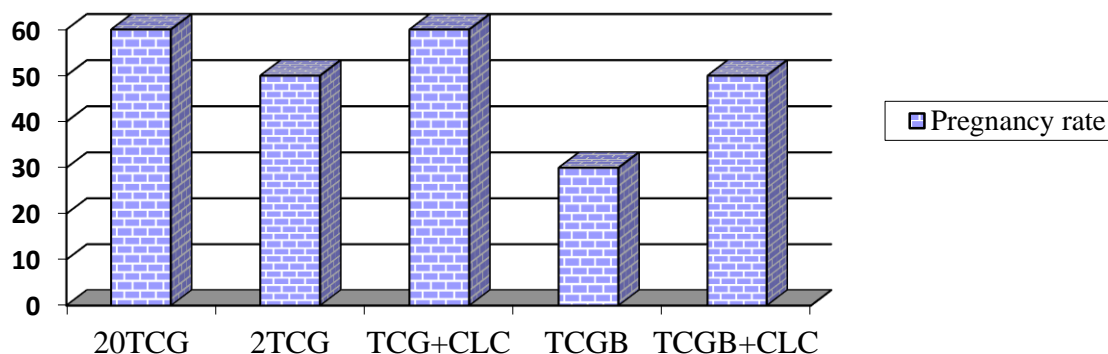


Figure 2 Effect of CLC and BSA on the pregnancy rate after insemination with frozen-thawed semen in goats (n=10/ group). No significantly differences between the percentage of the treatments at $p < 0.05$.

The main findings of our study clearly indicated that treating goat spermatozoa with albumin or albumin with CLC prior cryopreservation significantly decreased the percentage of total motility, progressive motility, viability, normal sperm morphology, and membrane integrity after thawing. The BSA in semen extender had adsorption on liposome as likely to be related to the hydrophobic interaction between BSA and liposomes. It is found that micro-fluidity of liposomal bilayer membranes near the bilayer center decreased by adsorption of BSA (Yokouchi et al., 2001). Santos et al. (2007) who studies cryopreservation of dog spermatozoa reported lower acrosome integrity and viability in tris extender containing BSA as well as tris extender containing egg yolk. The effects of albumin on the viability and fertilizing ability of sperm are controversial and may be dissimilar across species. In some species, BSA can increase the fowl spermatozoa fertility when the overdose of BSA (16 mg/ml) is used (Blesbois et al., 1992).

There were no significant differences of pregnancy rate between the treatments, standard and control group but tended to have higher pregnancy rate (50-60%) in the standard group and second group (extender with CLC) when compared with others (30%). The estrus does were transcervical inseminated with $20\text{-}40 \times 10^6$ motile sperm that could make pregnant. In general, the pregnancy rate by transcervical insemination with frozen semen of motile sperm was approximate 32-64% (Arrebola et al., 2012). The recommended a minimum number of only 20×10^6 motile sperm while there are several reports of acceptable fertility (>50%) using doses as low as 5×10^6 and 10×10^6 motile sperm that could produce pregnancy of does (El-Badry et al., 2014).

In conclusion, semen extender containing CLC was the key element in the improvement of the sperm

motion, kinetics, viability and membrane integrity of buck sperms. Moreover, the technique proves to be simple, and it can be readily utilized in currently used protocols for the freezing of goat semen in the field practice.

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บทคัดย่อ

คุณภาพน้ำเชื้อแพะภายหลังการแช่แข็งโดยใช้อัลบูมินและคอเลสเตอรอลทดแทนไข่แดง ในสารเจือจางน้ำเชื้อ

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การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาผลของอัลบูมินและคอเลสเตอรอล เพื่อทดแทนการใช้ไข่แดงในสารเจือจางน้ำเชื้อแพะต่อคุณภาพน้ำเชื้อแพะภายหลังการแช่แข็งแล้วทำละลาย และอัตราการการตั้งท้อง ทำการรีดเก็บน้ำเชื้อโดยใช้ช่องคลอดเทียมจากพ่อพันธุ์แพะจำนวน 3 ตัว แบ่งน้ำเชื้อออกเป็นการทดลอง 5 กลุ่ม คือ กลุ่มที่ 1 ทำการปั่นแยกเซอุมินอลพลาสมาออกและทำการเติม สารเจือจางน้ำเชื้อร่วมกับไข่แดง 20% (กลุ่มมาตรฐาน) กลุ่มที่ 2 ใช้น้ำเชื้อทั้งหมดเติมสารเจือจางน้ำเชื้อร่วมกับไข่แดง 2% (กลุ่มควบคุม) กลุ่มที่ 3 ใช้น้ำเชื้อทั้งหมดเติมสารเจือจางน้ำเชื้อร่วมกับ อัลบูมิน 4 mg/mL กลุ่มที่ 4 ใช้น้ำเชื้อทั้งหมดเติมสารเจือจางน้ำเชื้อร่วมกับคอเลสเตอรอล 5 mg/mL กลุ่มที่ 5 ใช้น้ำเชื้อทั้งหมดเติมสารเจือจางน้ำเชื้อ ร่วมกับอัลบูมิน 4 mg/mL และคอเลสเตอรอล 5 mg/mL นำน้ำเชื้อที่ได้บรรจุในหลอดขนาด 0.25 mL จากนั้นทำการแช่แข็งด้วยเครื่องลดอุณหภูมิอัตโนมัติและเก็บในไนโตรเจนเหลว ภายหลังจากแช่แข็งทำการละลายน้ำเชื้อที่อุณหภูมิ 37°C นาน 30 วินาที ผลพบว่า กลุ่มที่ทำการเสริมด้วยคอเลสเตอรอลให้ผลที่เหมือนกับกลุ่มควบคุมมาตรฐาน ส่วนกลุ่มที่เสริมด้วยอัลบูมินหรือกลุ่มที่เสริมด้วยอัลบูมินร่วมกับคอเลสเตอรอล มีความแตกต่างจากกลุ่มควบคุมทั้งสองอย่างมีนัยสำคัญทางสถิติ ($P < 0.05$) จากการประเมินคุณภาพน้ำเชื้อด้วยกล้องจุลทรรศน์ และการประเมินคุณภาพน้ำเชื้อด้วยเครื่องคอมพิวเตอร์ การผสมเทียมเพื่อดูอัตราการผสมติดพบว่า กลุ่มที่ทำการเสริมอัลบูมินมีแนวโน้มของอัตราการตั้งท้องต่ำกว่ากลุ่มอื่น แต่ไม่มีความแตกต่างกันทางสถิติ สรุปได้ว่า การเสริมคอเลสเตอรอลเพื่อทดแทนไข่แดงในสารเจือจางน้ำเชื้อแพะได้ผลดี และสามารถใช้น้ำเชื้อแพะสำหรับการแช่แข็งน้ำเชื้อและใช้ได้จริงในภาคสนาม

คำสำคัญ: การแช่แข็งน้ำเชื้อ แพะ อัลบูมิน คอเลสเตอรอล ไข่แดง

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