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Effect of Various Coconut (*Cocos nucifera*) Milk Concentrations Instead of Egg Yolk on Freezability and Fertility of Frozen Holstein Bull Spermatozoa

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The study was carried out to investigate if the substitution of egg yolk (EY) with various coconut (*Cocos nucifera*) milk concentrations in extenders can improve the freezability and fertility of frozen Holstein bull spermatozoa. Semen from five Holstein bulls was collected twice weekly and ejaculates with 75% progressive motility and more 85% normal sperm morphology prior to cryopreservation were pooled in order to have sufficient semen for a replicate and to eliminate the bull effect. Five extenders were used. Tris 20% egg yolk extender with 7% glycerol as a control, and substitution of whole egg yolk with 5, 10, 15, and 20% coconut milk. Semen was diluted to 80 million sperm/ml packaged into 0.25ml straws, cooled held at 5°C for 4 h, and then frozen in liquid nitrogen and stored at –196°C until for artificial insemination. Sperm progressive motility, live sperm, sperm abnormality, intact sperm acrosome and plasma membrane integrity were evaluated post dilution, post–equilibration and post frozen–thawed processes. The results revealed that 5% coconut milk extender was more effective in preservation of progressive motility, live sperm, sperm abnormality, intact sperm acrosome and plasma membrane integrity of Holstein spermatozoa than whole egg yolk extender and other coconut milk extenders. Fertility rates were higher in 5% and 10% coconut milk extenders compared with whole egg yolk extender (65% and 55% vs 45%, respectively) and other 15% and 20% coconut milk extenders (45% and 40%, respectively). It was concluded that 5% coconut milk extender improved the freezability and fertility of Holstein bull spermatozoa.

Key words: Holstein Bull Semen, Coconut Milk, Freezing, Fertility

INTRODUCTION

Sperm cryopreservation is the most efficient method for storing bull spermatozoa for long period even though their fertilizing ability is still, lower than that of fresh or liquid–preserved semen (Hammerstedt *et al*., 1990). Successful semen cryopreservation depends on the use of suitable extender, cryoprotectant and the proper cooling/warming process (Fiser, 1991). Mainly, the cryoprotectant plays a major role in this procedure by resisting sudden temperature change, protecting sperm against cold and hot shock damage (Watson, 1999), as well as preventing ice formation during freezing–thawing process. However, Morris *et al*. (2007) reported that intracellular ice formation does not induce significant cryodamage, and limited water was observed in sperm. Egg yolk is frequently used as a cryoprotectant agent in mammalian semen diluted and showed to be highly effective for the maintenance of sperm fertility stages (Sansone *et al*., 2000; Garde *et al*., 2003). It is believed that the beneficial effect of EY during cryopreservation process can be attributed to phospholipids, cholesterol low density lipoproteins which protecting sperm against cold shock (Moussa *et al*., 2002; El–Sharawy *et al*., 2012[a, b]). Norman (1962) is the first used coconut milk as part of extender for semen and who added also coconut milk had the advantage of keeping livability of fresh semen. In goat semen, Melo and Nunes (1991) reported that semen extended in coconut milk led to give appreciable sperm cell motility and fertility with acceptable conception rate post–breeding. Coconut milk is contained energy compounds such as sugars (glucose, fructose, lactose, and sucrose), fatty acids, proteins and amino acids (glutamic acid, glutamine, alanine, arginine, lysine,leucine, proline, etc), minerals and trace elements (Na^+, $Ca2^+$, $K2^+$, $Mg2^+$, Cl^-, $Fe3^+$, P, N2, $Co2^+$, $Zn2^+$, $PO4^-$, HCO3 – etc), vitamins, antioxidant enzymes (catalase, peroxidase), phospholipids, and phytohormones (cytokinins) for example kinetin reputed to have ant stress, anti–ageing, anti–carcinogenic and anti–thrombotic effects (Abara *et al*., 2007; DebMandal and Mandal, 2011; Solangi and Iqbal, 2011). Sule *et al*. (2007) reported that coconut milk has been employed in various proportions and in varying combinations in semen extenders for storage (hours to days) under controlled and ambient conditions. However, to our knowledge, no report has evaluated the effect of coconut milk in extender on freezability of bull semen under –196°C in liquid nitrogen for long storing.

Therefore, the present study was carried out to evaluate and compare the effect of substitution of EY with coconut milk on bull semen cryopreservation and on subsequent fertilization.

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MATERIALS AND METHODS

Experimental animals:

This study was conducted for a periods of 8 weeks. Five adult Holstein bulls, aged (3–5 years) with clinically normal reproductive tract and kept at the International Livestock Management Training Center (ILMTC), Sakha, belonging to the Animal Production Research Institute, Agricultural Research Center, Ministry of Agriculture. The bulls included herein were regular semen donors with satisfactory semen quality and having good fertility records.

Semen collection:

Semen was collected twice weekly, using an artificial vagina, from each bull and immediately held in a water bath adjusted at 37°C and evaluated for sperm motility. Ejaculates showing at least 75% sperm motility and more than 85%normal spermatozoa were selected for further processing. On each collection day, good ejaculates were pooled and divided into 5 parts; the first was diluted with Tris–20%EY (control), while the other four parts were diluted with Tris extender without EY supplemented with coconut milk at levels of 5, 10, 15 and 20%, respectively.

Preparation of semen extenders:

Tris–based extender: 3.025 g Tris– (hydroxymethyl–aminomethane), 1.675 g citric acid, 0.75 g glucose and 7.0% glycerol, 20% egg, 0.25 g lincomycin and 0.05 g streptomycin and completed with bi–distilled water up to 100 ml.

Coconut milk: The preparation of coconut milk extender followed a simple but aseptic procedure. The meat of freshly harvested coconut (*Cocos nucifera*) was thoroughly blended and collected in a 250 ml conical flask. The water from the coconut was added to the blend and the mixture allowed standing for about 1 h. Thereafter, the mixture was wrapped in a heat sterilized white cloth and tightly squeezed to express the milk. The milk was filtered through sterilized white clothes thrice to get rid of all residues and the liquid was collected in a sterilized flask. The whole 20% EY in Tris–based extender was replaced by different concentrations of 5,10,15, and 20% coconut milk extraction.

Extension and freezing semen:

Each pooled semen sample was spilt into five aliquots and extended at 37°C with one of five experimental extenders to achieve the final concentration of 80 million spermatozoa per ml of the extended semen. Immediately after extension, the percentage of progressive motility, live sperm, sperm abnormality, acrosome and plasma membrane integrity were recorded. Then, extended semen was maintained in a refrigerator at 5°C for 4 h for equilibration. The samples were filled in 0.25 ml French straws with the help of an automatic suction machine. Subsequently, these straws were placed 4cm above the liquid nitrogen surface where the temperature was approximately –120°C. After 10 min all straws were immersed directly into liquid nitrogen at –196°C for freezing. Also, the percentages of progressive motility, live sperm, sperm abnormality, intact acrosome integrity and plasma membrane integrity were evaluated post–equilibration and post –thawing processes.

Thawing frozen semen:

The straws containing the EY, 5%, 10%, 15% and 20% coconut milk extenders were plunged directly into water bath at 37°C for 30 Sec, then wiped with absorbent paper. The ends were then cut and the contents emptied into a plain tube maintained at 37°C and were analyzed 10min later. Progressive motility was estimated according to Melrose and Laing (1970) live and abnormalities were estimated according to Hancock (1951) acrosome and plasma membrane integrity were determined according to Kovacs and Foote (1992) and Jeyendran *et al.* (1984) respectively.

Fertility trail:

A total of 100 Holstein cows were artificial inseminated with random frozen doses from five various extenders (20 cow each). Each cow was inseminated with a single straw 8–14 after start of estrus behavior. Using rectovaginal technique and the universal insemination gun, the frozen–thawed semen was deposited in the uterine body just next to the anterior end of the cervix. Conception rate was calculated on the basis of pregnancies confirmed by rectal palpation 45–60 day after insemination.

Statistical analysis

Data were statistically analyzed by the methods of analysis of variance according to model procedures of SPSS (2013). Duncan Multiple Range Test was used to test the differences among means (Duncan, 1955).

RESULTS

The effect of different concentrations of coconut milk in Tris–extender and Tris–20% egg on frozen Holstein sperm quality parameters are shown in Tables 1, 2, 3, 4 and 5.

The progressive motility of Holstein spermatozoa in 5% coconut milk extender was equal with that in control Tris–egg yolk extender post dilution (72.1–72.3%, respectively) but it was significantly ($P<0.05$) higher during post equilibration and post frozen–thawed processes (66±0.69% and 57.3±0.99 % vs 60.5±1.19 % and 48.5±1.21 respectively (Table 1). In addition, the progressive motility of spermatozoa in 10% and 15% coconut milk extenders were higher after equilibration and post frozen–thawed processes compared to that in control Tris–egg yolk extender and 20% coconut milk extender. The 20% coconut milk showed the lower progressive sperm motility during post–dilution, equilibration and thawing processes than all other extenders.

The percentage of live Holstein spermatozoa was improved in 5%, 10%, and 15% coconut milk extenders post equilibration and post frozen–thawed processes

compared to control and 20% coconut milk extenders (Table 2) In addition, the increasing coconut milk to 20% led to decrease the percentage of sperm livability post three stages of cryopreservation compared to other extenders.

The percentage of sperm abnormality was lower only in semen extended with 5% coconut milk extender compared to control and other coconut milk extenders during post–dilution, post– equilibration and post frozen–thawed processes (Table 3) The highest sperm abnormality was observed in 10%, 15% and 20% coconut milk extenders compared to 5% coconut milk and control (Tris–egg yolk extenders).

The percentage of sperm plasma membrane integrity in 5% coconut milk extender was significantly ($P<0.05$) higher than control and other coconut milk extenders

Table 1. Effect of Tris–EY and coconut milk extenders on progressive motility (%) of Holstein bull spermatozoa at different stages of cryopreservation. (Mean±S.E)

Item	Tris–EY* (Control)	Tris–Coconut milk levels (%)			
		5	10	15	20
Post dilution	72.3^{a}±0.77	72.1^{a}±0.79	68.0^{b}±1.11	67.5^{b}±0.92	63.0^{c}±0.67
Post equilibration	60.5^{b}±1.19	66^{a} ±0.69	62.3^{b}±0.99	60.8^{b}±1.32	54.0^{c}±1.24
Post thawing	48.5^{c}±1.21	57.3^{a}±0.99	53.8^{b}±0.99	49.8^{c}±1.33	42.5^{d}±1.23

*Tris–EY: Tris Egg Yolk Extender

a, b, c and d: the different superscripts in the same row are significant at ($P<0.05$).

Table 2. Effect of Tris–EY and coconut milk extenders on live spermatozoa (%) of Holstein bull spermatozoa at different stages of cryopreservation. (Mean±S.E)

Item	Tris–EY* (Control)	Tris–Coconut milk levels (%)			
		5	10	15	20
Post dilution	76.3^{a}±0.81	76.3^{a}±0.69	73.3^{b}±1.03	71.1^{b}±1.06	67.0^{c}±0.73
Post equilibration	64.9^{b}±1.03	69.9^{a}±0.80	66.1^{b}±1.19	65.2^{b}±1.29	57.4^{c}±1.45
Post thawing	52.5^{c}±1.01	61.5^{a}±1.00	57.7^{b}±0.89	53.9^{c}±1.46	47.5^{d}±1.24

*Tris–EY: Tris Egg Yolk Extender.

a, b, c and d: the different superscripts in the same row are significant at ($P<0.05$).

Table 3. Effect of Tris–EY and coconut milk extenders on abnormality (%) of Holstein bulls spermatozoa at different stages of cryopreservation. (Mean±S.E)

Item	Tris–EY* (Control)	Tris–Coconut milk levels (%)			
		5	10	15	20
Post dilution	7.1^{c}±0.14	7.2^{bc}±0.14	7.8^{a} ±0.12	7.5^{abc}±0.17	7.6^{ab}±0.14
Post equilibration	8.0^{c}±0.12	8.8^{c} ±0.20	9.0^{ab}±0.18	9.1^{ab}±0.26	9.5^{a} ±0.27
Post thawing	10.2^{c}±0.23	10.9^{bc}±0.26	11.3^{ab}±0.19	11.4^{ab}±0.24	12.0^{a} ±0.32

*Tris–EY: Tris Egg Yolk Extender.

a, b and c: the different superscripts in the same row are significant at ($P<0.05$).

Table 4. Effect of Tris–EY and coconut milk extenders on plasma membrane integrity (%) (Mean±S.E) of Holstein bull spermatozoa, at different stages of cryopreservation

Item	Tris–EY* (Control)	Tris–Coconut milk levels (%)			
		5	10	15	20
Post dilution	74.8^{a}±0.63	73.7^{a}±0.72	70.6^{b}±1.02	68.7^{b}±0.86	65.0^{c} ±0.55
Post equilibration	63.9^{b}±0.80	67.9^{a}±0.89	63.2^{b}±0.95	63.4^{b}±1.11	57.2^{c} ±1.29
Post thawing	51.5^{c}±0.94	59.4^{a}±1.03	56.0^{b}±0.82	52.4^{c}±1.30	45.45^{d}±1.27

*Tris–EY: Tris Egg Yolk Extender.

a, b, c and d: the different superscripts in the same row are significant at ($P<0.05$).

Table 5. Effect of Tris–EY and coconut milk extenders on acrosome integrity (%) (Mean±S.E) of Holstein bull spermatozoa, at different stages of cryopreservation

Item	Tris–EY* (Control)	Tris–Coconut milk levels (%)			
		5	10	15	20
Post dilution	73.65^{ab}±0.58	72.31^{a}±0.76	69.20^{b}±0.99	67.95^{b}±0.92	63.80^{c}±0.76
Post equilibration	63.80^{ab}±1.02	66.75^{a}±0.90	61.30^{b}±1.07	61.70^{b}±1.20	56.35^{c}±1.15
Post thawing	51.30^{c} ±1.06	58.45^{a}±0.94	54.65^{b}±0.87	50.90^{c}±1.45	44.90^{d}±1.29

*Tris–EY: Tris Egg Yolk Extender.
a, b, c and d: the different superscripts in the same row are significant at ($P<0.05$).

Table 6. Effect of Tris–EY and coconut milk extenders on conception rate of Holstein

Item	Tris–EY* (Control)	Tris–Coconut milk levels (%)			
		5	10	15	20
Inseminated animals	20	20	20	20	20
Conceived animals	9	13	11	9	8
Conception rate (%)	45	65	55	45	40

*Tris–EY: Tris Egg Yolk Extender.

during equilibration and post –thawing processes (Table 4). In addition, semen extended with 10% and 15% coconut milk extenders improved plasma membrane integrity at post– equilibration and post –thawing processes compared to that in control Tris–egg yolk extender and 20% coconut milk extender. Moreover, increasing coconut milk in semen extended to 20% led to lower ($P<0.05$) plasma membrane integrity compared to control Tris–egg yolk extenders during all stages of cryopreservation. The trend of results observed in intact sperm acrosome (Table 5) was the same with the previous percentage of sperm plasma membrane integrity.

Conception rate:

The effect of different coconut milk extenders and Tris–egg yolk extender on conception rate are shown in Table 6. Semen extended with 5% and 10% coconut milk extenders improved conception rate to 65% and 55%, compared to that in control–Tris egg yolk extender and 15% or 20% coconut milk extender (45% and 45% or 40% respectively).

DISCUSSION

The present findings indicated that using coconut milk in semen extender improved the quality of cryopreserved spermatozoa of Holstein bull and its subsequent fertility. The present results demonstrated coconut milk extenders (except level 20%) provided a more adequate medium to sustain the progressive motility, live sperm, intact plasma membrane and intact acrosome of Holstein spermatozoa cryopreserved as evidenced from its ability to maintain sperm parameters better than the control . This improvement observed on sperm frozen–thawed parameters indicated that coconut milk extenders (5%, 10% and 15%) contained essential constituents such as sugars, vitamins, minerals and amino acids (Yong *et al* ., 2009; USDA National Nutrient Database, 2015) required for cryosurvival of spermatozoa.

Many authors confirmed that the role of sugars as a source of energy, an osmolyte and a cryoprotectant of sperm survival following cryopreservation process (Yancy, 2005; Purdy, 2006; Naing *et al*., 2010; Daramola *et al*., 2016[a]). In goat semen, Koshimoto and Mazur (2002) and Aboagla and Terada (2003) suggested that goat spermatozoa readily utilizes sugar for respiration, and these sugars also provide osmotic balance and cryopreservation. The reduction in sperm motility, live sperm, plasma membrane integrity and acrosome integrity in various coconut milk extenders during post dilution process might been caused by an increase in the number of sperm abnormalities.

The protective effect of coconut milk also be linked to its high content of lipids (Yang *et al*., 2009) which a major component of sperm membrane that is involved in a series of biochemical and functional change ultimately required for fertilization (Brque *et al*., 2003).

During cryopreservation, significant changes in lipid composition and phospholipids loss in seminal plasma occur (Chakrabarty *et al*., 2007; Futino *et al*., 2010) leaving sperm vulnerable to oxidative stress due to of antioxidant protection (Bucak *et al*., 2010). Therefore, the exogenous phospholipids present in extenders derived from coconut milk possibly replaced some of the sperm membrane phospholipids lost during cryopreservation to maintain the plasma membrane structure and function (Zhang *et al*., 2009). Moreover, phospholipids play important physiological function in reducing the freezing point, avoiding the formation of large ice crystals and resulting in reduced mechanical damage to sperm membrane (Giraud *et al*., 2000; Waterhouse *et al*., 2006; Daramola *et al*., 2016[b]). Furthermore, the protective

effect of coconut milk could also be attributed to its major proteins including essential amino acids which play an important role in cell membrane integrity (Sakanaba *et al*., 2004; Yang *et al*., 2009). The cryoprotective effect of amino acids in mammalian spermatozoa during freezing stemmed from their ability to form a layer on the spermatozoa surface, and positively charged molecules combined with the phosphate groups of sperm plasma membrane phospholipids has been observed (Kundu *et al*., 2001; Atessahin *et al*., 2008).

In addition, the improved frozen–thawed of Holstein spermatozoa in the present study with 5% coconut milk extender indicated the ability in coconut milk extender to efficiency harness the potassium contained in coconut milk and spermatozoa for survival of the spermatozoa during cryopreservation (Okolie *et al*., 2011). 20% coconut milk extender was the poorest semen extender probably on coconut milk of its high viscosity which impeded sperm motility (Sule *et al*., 2007; Okukpe *et al*., 2012).

The present improvement in cryopreserved spermatozoa was closed with that reported by Daramola *et al*. (2016[a,b]) in buck semen. Conception rate in cow inseminated with semen cryopreserved in extenders containing different coconut milk concentrations (5% and 10%) were higher than control and other 15% or 20% coconut milk extenders (65% and 55% vs 45% and 45% or 40%, respectively) see Table 6. It showed be mentioned that conception rate achieved in the present study (55% to 65%) is satisfactory as compared with the previous studies using freezing and thawing techniques.,

Based on the foregoing results, it could be concluded that coconut milk extender process remarkable cryoprotective properties for freezing Holstein bull spermatozoa. Higher frozen–semen quality and conception rate were achieved with the use of 5% and 10% coconut milk extenders as compared to 20% egg yolk semen extender.

AUTHOR CONTRIBUTIONS

1) Study conception and design: I. EL–SHAMAA; R. MAHMOUD; E. EL–SIEFY and M. IBRAHIM
2) Acquisition of data: R. MAHMOUD; E. EL–SIEFY; I. M. IBRAHIM and I. EL–SHAMAA
3) Analysis and interpretation of data: E. EL–SEIFY; M. EL–SHARAWY; N. YAMAUCHI and I. EL–SHAMAA
4) Drafting of manuscript: I. EL–SHAMAA; R. MAHMOUD; E. EL–SIEFY; M. EL–SHARAWY; N. YAMAUCHI and K. KUBOTA
5) Critical revision: M. EL–SHARAWY; M. IBRAHIM; K. KUBOTA; N. YAMAUCHI and I. EL–SHAMAA

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