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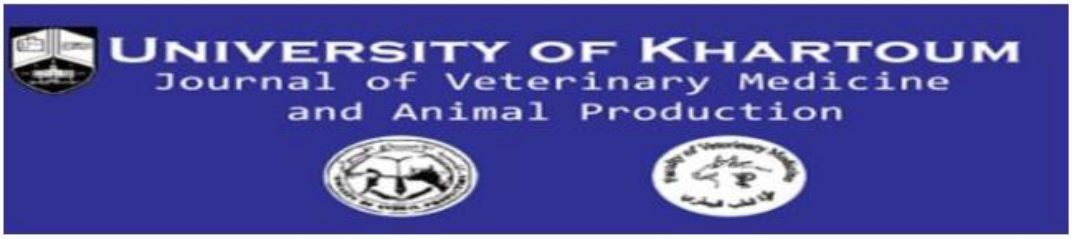

Effect of Virgin Coconut Oil Combined with Coconut Milk on the Quality of Chilled and Frozen-Thawed Bull Semen

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Abstract

The aim of this study was to assess the effects of virgin coconut oil (VCO) combined with coconut milk (CM) on chilled and frozen–thawed quality parameters of bull sperm. Twenty four semen samples were collected from four bulls. Samples were diluted in Tris extender containing 0% (control only 3% CM) in Tris-base extender), 2%, 4%, 6%, 8% VCO mixed with 3% CM and 20% egg yolk (group B). Extended samples were divided into two fractions, one was chilled at 4°C for 24, 72 and 144 hours and the second fraction packed into 0.25 ml straws and frozen in liquid nitrogen for 7 and 14 days. Subsequently, chilled samples and the straws were thawed and evaluated for sperm motility by Computer Assisted Semen Analyzer (CASA), viability, acrosome integrity, morphology (eosin-nigrosin) and membrane integrity (hypo-osmotic swelling test). Higher ($p < 0.05$) percentage of sperm parameters was observed in the positive control group in chilled semen, followed by 8% VCO compared to the other concentrations of the VCO. There was no significant difference in morphology between positive control and all other treatment groups in chilled or frozen-thawed semen. The positive control group was higher in all sperm parameters measured when compared with treated groups in frozen-thawed semen. In conclusion the results showed that the mixture of VCO with coconut milk applied in this study could not maintain the function of bull sperm after chilled and frozen-thawed better than the positive control.

Keywords: Virgin Coconut Oil, Coconut milk, Bull semen, Quality parameters, Chilled; Frozen

المستخلص

ان الهدف من هذه الدراسة هو تقييم تأثير زيت جوز الهند البكر (VCO) مع حليب جوز الهند (CM) على مقاييس جودة الحيوانات المنوية المبردة والمجمدة بعد الاذابة. جمعت أربع وعشرون عينة من السائل المنوي من أربعة ثيران. تم تخفيف العينات في مخفف Tris-base extender كممدد أساسي للحجم وكانت المعاملات كالتالي: مجموعة التحكم السالب (أ) والخالية (0%) من مخلوط زيت جوز الهند البكر وحليب جوز الهند (فقط 3٪ من حليب جوز الهند مع المخفف الاساسي) ، أما مجموعة التحكم الموجب (ب) فتحتوي على 2٪ ، 4٪ ، 6٪ ، 8٪ من مخلوط زيت جوز الهند البكر وحليب جوز الهند مع المخفف الاساسي اما المجموعة المعالجة (ج) فكانت تحتوي على 20٪ صفار بيض مع المخفف الاساسي. تم تقسيم كل العينات المخففة إلى جزئين ، أحدهما تم تبريده عند 4 درجات مئوية لمدة 24 و 72 و 144 ساعة والجزء الثاني تمت تعبئته في قصبات تلقيح بحجم 0,25 مل و من ثم حفظه بالتجميد في النيتروجين السائل (- 196 °C) لمدة 7 و 14 يومًا. بعد ذلك ، تم إحماء العينات المبردة وإذابة قصبات التلقيح وتقييمها من أجل تقييم حركة الحيوانات المنوية بواسطة جهاز (CASA)، اما قياس الحيوية ، وسلامة الجسيمات ، والشكل الظاهري للحيوانات المنوية فتم باستخدام صبغة (eosin-nigrosin) وسلامة الغشاء الخارجي ب(hypo-osmotic swelling test). في السائل المنوي المبرد لوحظ وجود النسبة الاعلى ($p < 0.05$) من مقاييس جودة الحيوانات المنوية في المجموعة (ب) ، تليها مجموعة 8٪ من مخلوط زيت جوز الهند البكر مع حليب جوز الهند في المجموعة (ج) مقارنة بالتراكيز الأقل منه .لم يكن هناك فرق معنوي في شكل الحيوان المنوي بين مجموعة الكونترول الموجب وجميع المجموعات الأخرى في السائل المنوي المبرد أو المجمد. كانت المجموعة (ب) أعلى في جميع مقاييس جودة الحيوانات المنوية التي تم قياسها عند مقارنتها بالمجموعات الأخرى في السائل المنوي المجمد بعد الاذابة. في الختام أظهرت النتائج أن خليط زيت جوز الهند البكرمع حليب جوز الهند المطبق في هذه الدراسة لا يمكن أن يحافظ على وظيفة الحيوانات المنوية للثور بعد تبريدها وتجميدها وتذويبها بشكل أفضل من الكونترول الموجب.

الكلمات المفتاحية: زيت جوز الهند البكر، حليب جوز الهند، السائل المنوي للثيران، صفات الجودة، مبرد، مجمد.

Introduction

Egg yolk is one of the important constituents of semen extenders that provide protection to sperm against cold shock during cryopreservation (Zhang *et al.,* 2009). However, some researchers considered those animal origin components might introduce risks of microbial contamination during AI procedure of farm animals (Aires *et al*., 2003; Bousseau *et al*., 1998). According to Aires *et al*. (2003), the risk of microbial contamination associated with egg yolk reduced the fertilizing capacity of bovine spermatozoa. Moreover, according to World Organization for Animal Health (OIE, 2003), the semen extender constituents is recommended to be free of any a microbiological risk (Marco-Jimenez *et al*., 2004). These situations require substituting the egg yolk partly or completely with extenders of vegetable origin, which is conceived to be alternative to milk or egg yolk based extenders (Gil *et al*., 2003).

Several commercial extenders using non-animal origin extenders have shown promising results in different animal species on various quality assessment parameters (Rehman *et al*., 2014). With cryopreserved semen, there is a noticeable decrease in sperm movement, viability and progressive motility in the female reproductive tract, which resulted in fertility reduction (Barbas and Mascarenhas, 2009). It has been indicated that the cryopreservation procedure induced formation of reactive oxygen species (ROS) (Watson, 1995), which caused loss of sperm plasma membrane function and subsequently reduced sperm survival and fertilizing ability (Aitken, 1995). Watson (2000) reported that, impairment of membrane function is mainly caused by the change of lipids into a rigid structure during cryopreservation. Polyunsaturated fatty acids (PUFA) represent the main components of cell plasma membrane (Conquer *et al.*, 1999) and addtion of PUFA plays a significant role in the protection of sperm cell membrane during freezing and thawing (Robinson *et al*., 2006). Kaka, *et al* (2015c) reported that addition of 3 ng/ml docosahexanoicacid (DHA) to BioXcell®

extender enhanced the quality of cryopreserved bull semen. Furthermore, they also showed that supplementation of linolenic and α-linolenicacid in Bioxcell® and Tris extenders enhanced the quality of cryopreserved bovine semen (Kaka, *et al.*, 2015a; 2015b). Moreover,Hossain *et al.*(2007) and Kiernan,(2012) reported that semen quality parameters of chilled and cryopreserved semen of boars and bulls were enhanced when palmitic acid, linoleic acid, oleic acid and arachidonic acid were added. According to Aires et al. (2003), plant lipid sources have been used to replace the egg yolk and to reduce the hazards of animal disease transmission. One of these plant lipid or oils is VCO. Virgin coconut oil contains saturated and unsaturated fatty acids (Dosumu *et al*., 2010). Lauric acid represents 50% of the total fatty acids, which is considered as antibacterial, antiviral, antinociceptive and anti-inflammatory component (Zakaria *et al.*, 2011). Moreover, VCO is rich in antioxidants such as tocotrienol, polyphenols, and tocopherols (Marina et al., 2009a; Nevin and Rajamohan, 2006). Furthermore, according to Gutierrez et al. (2006), coconut milk contributed to suitable outcomes of the sperm parameters when used as cryoprotectant in ram semen extender. Depending on these properties of VCO, a hypothesis was formulated that VCO combined with coconut milk (lecithin) can be used as substitute to egg yolk for chilling and freezing bull semen. Therefore, this experiment was designed to determine the effects of adding VCO combined with coconut milk to Tris-based semen extender on chilled and cryopreserved bull semen quality parameters.

Materials and methods

Animals

Four healthy, sexually matured and fertile crossbred bulls (2 Brangus- Friesian) and (2 Brangus-Simmental) were selected for that study based on their farm reproductive records. The bulls are 3 to 5.5 years old and weighed between 600 - 650 kg. All bulls were kept in the bull pens and managed under similar management condition. They were fed with *Brachariadecumbens* as green fodder. Commercial Palm Kernel Cake (16% crude protein and 2.6% crude fat) was given as supplements, at 3 kg per bull daily. Water and salt licks were provided *ad libitum*.

Semen collection and experimental design

A total of 24 semen samples were collected using an electro ejaculator (Electro- Jac6, Ideal Instruments - Neogen Company, Lexington- KY, USA) twice a week. Semen samples were transported to the laboratory at 37°C for initial evaluation tests. Samples with total motility of ≥70%, normal morphology and viability percentage of ≥80% were selected for further processing of the experiment.

Preparation of the extenders

Two Tris–Citric acid based semen extenders were prepared as reported earlier (Amirat *et al.*, 2010). Briefly, it contained Tris (2.42 g), fructose (1.00 g), citric acid (1.48 g), glycerol (6.4%), egg yolk (20%) and distilled water up to 100 ml. In the treatment group, egg yolk was replaced with different concentrations 2%, 4%, 6% and 8% of virgin coconut oil (VCO; Nano XanSdn. Bhd and Malaysia Agriculture Research and Development Institute, Malaysia) as indicated in Table 1.

The VCO is hydrophobic and therefore cannot dissolve by itself in the extender. However, oil in water emulsions as VCO is known to be emulsified by lecithin.

The lecithin is reported to be an excellent agent (Dickinson, 1993), it functions without any known side effect on a biological system (semen). Coconut milk (CM) was used as a natural source of lecithin (Birosel et al., 1963). Since VCO is

insoluble in water (as lipid). 10 ml stock solution was prepared from blends of 3ml coconut milk (Ayam Brand-Malaysia.Ayam Brand Trim Coconut Milk) and 7 ml VCO the blend was shaken well utilizing a small scale shaker and a Stirrer.The extender was separated into two parts.The first part was used for chilling it was without glycerol, and divided according to number of VCO conentrations.The second part was employed for freezing, it was VCO and it contained glycerol. Diluted samples intended for chilling were kept in a refrigerator at 4°C and assessed after 24, 72 and 144 hours of storage for sperm motility, viability, morphology, membrane integrity and acrosome integrity. Samples intended for freezing were first Chilled for 4 hours at 4°C, and then stuffed in 0.25 ml straws at 20×10^6 sperm/straws frozen according to (Khumran *et al.*, 2015) and finally stored in liquid nitrogen. The frozen samples were thawed at 37°C for 30 seconds following 7 and 14 days of storage and evaluated for sperm motility, viability, morphology, membrane integrity and acrosome integrity.

Table 1 Design of semen extender (VCO+ coconut milk) used

Treatment groups	**Components of extender**
Positive control (C+)	Tris-based (20% egg yolk) extender (Amirat et al., 2010)
Negative control (C-)	Tris + 3% coconut milk
Treatment 1	Tris + 2% (VCO + coconut milk)
Treatment 2	Tris + 4% (VCO + coconut milk)
Treatment 3	Tris + 6% (VCO + coconut milk)
Treatment 4	Tris + 8% (VCO + coconut milk)

Sperm Motility, Morphology and Viability

Total sperm motility was evaluated utilizing a Computer Assisted Semen Analyzer (CASA) (Hamilton-Thorne Bioscience, USA). A total of 10 µl of semen sample put on a slide warmer and secured with a cover-slip, after that the slides were put in the CASA and the motility was recorded. Sperm viability and morphology were evaluated using eosin-nigrosin stain (Khumran *et al.*, 2015). Spermatozoa viability was assessed at 400 X magnification under a light microscope (Nikon Eclipse 50i, Tokyo-Japan). Total of 200 sperm were counted and sperm that stained white or pink color were evaluated as alive and dead, respectively (Memon *et al.*, 2012).

Plasma membrane integrity

Hypo-osmotic swelling test (HOST) was used to examine plasma membrane integrity(*Kaka et al*., 2015). One hundred microliters of semen was added to 1ml of hypo-osmotic solution (13.51 g fructose and 7.35 g trisodium citrate dissolved in 1L distilled water; osmolarity of 150 mOsm/kg.) and incubated for 1h at 37 ◦C. Then, 15µl of the solution was placed on a prewarmed slide covered with a cover slip and sperms were evaluated under a light microscope at 400× magnification (Nikon Eclipse 50i, Tokyo, Japan). Spermatozoa that swelled in response to the test solution were considered normal cells. Two hundred spermatozoa per slide were counted from four different microscopic fields and expressed in percentage.

Acrosome integrity

Acrosome integrity was investigated using semen smear stained with eosin–nigrosin stain and examined under a phase contrast microscope at 1000× magnification oil

immersion (khumran *et al*., 2015). A total of 200 spermatozoa was tested for either detached or intact acrosome.

Statistical analysis

Data were analyzed using statistical software (SAS, 9.2 version), and the results were presented as mean ± standard error of the mean (SEM). Analysis of variance (two-way ANOVA) was utilized to evaluate the significance of treatment. Differences between the means were analyzed by using the Duncan's multiple range tests. The difference between treatments for each variable was considered statistically significant at $P<0.05$.

Results

The effect of different concentrations of VCO combined with coconut milk on the sperm parameters of chilled semen is presented in Table2. The results showed that at 24 hrs, 8% VCO has the greatest values than other VCO treated groups in general motility, membrane integrity and acrosome integrity and there were no significant difference in morphology and viability. However, the positive control group (20% egg yolk) has the highest values ($P<0.05$) than all VCO groups in all sperm parameters. There were no differences in morphology between treated and control groups during 24, 72 and 144 hrs of storage period. Subsequently, at 72 and 144 hrs of chilling all VCO treatment groups were decreased in general motility, viability and membrane integrity, however, 2% VCO treated group has better value of acrosome integrity among VCO treated groups at 72 and 144 hrs. The positive control group at 72 and 144 hrs was significantly ($P<0.05$) higher than all the VCO treatment groups in the most of sperm parameters except acrosome integrity at 144 hrs. Treatments are time sensitive; this is further reinforced by the fact there is a significant time treatment interaction.

Table.3 shows the effect of different concentrations of VCO combined with coconut milk on sperm parameters of frozen-thawed bull semen. The positive control group was significantly ($P<0.05$) higher at 7 and 14 days of the storage period in motility, viability, membrane integrity and acrosome integrity than all the VCO treatment groups. There were no significant differences among all the treatment groups and positive control in morphology parameter. Acrosome integrity in the VCO treated groups was better values than the other sperm parameters. The results showed that there were no significant differences in storage period (day 7 to day 14).

Discussion

In the present study, the results showed that the treated group with 8% VCO and coconut milk (CM) has better value of sperm parameters except viability when compared with other (VCO+CM) treatment groups after chilling for 24 hours. Moreover, the morphology and acrosome integrity were better values in the (VCO+CM) treated groups in chilled semen. However, the positive control group (20% egg yolk) was still the best extender with greater chilled sperm parameters for 24, 72 and 144 h.

Table 2: Effect of different concentrations of VCO combined with coconut milk in Tris-based extender on bull chilled sperm parameters at 24, 72 and 144 hours of storage (Mean ± SE).

Treatment (VCO + CM) %	Chilling time (hrs)	Parameter				
		Motility (%)	Morphology (%)	Viability (%)	Membrane integrity %	Acrosome integrity %
0% + 3% (C-)	24	38.50±7.72^{c}	98.08±0.47	42.83±6.80^{b}	36.67±7.56^{d}	81.50±2.47^{c}
2%	24	40.58±4.66^{c}	97.46±0.68	49.42±6.32^{b}	38.21±4.56^{cd}	86.13±1.79^{abc}
4%	24	53.67±6.^{67bc}	97.50±0.60	51.92±5.72^{b}	52.08±6.72^{bcd}	84.46±2.42^{bc}
6%	24	57.83±7.63^{bc}	97.83±0.46	53.50±7.45^{b}	56.08±7.56^{bc}	85.67±2.21^{abc}
8%	24	62.75±6.25^{b}	97.79±0.32	52.54±5.61^{b}	61.13±6.30^{b}	88.33±1.86^{ab}
20% egg yolk (C+)	24	89.58±4.54^{a}	98.25±0.43	81.38±3.82^{a}	91.88±2.82^{a}	91.38±1.00^{a}
0% + 3% (C-)	72	20.75±5.17^{b}	98.13±0.62	21.29±5.02^{b}	20.00±4.91^{b}	73.42±2.22^{b}
2%	72	15.50±3.61^{b}	98.17±0.25	25.38±5.10^{b}	14.21±3.53^{b}	82.29±2.10^{a}
4%	72	20.75±4.77^{b}	98.29±0.46	23.83±3.89^{b}	19.42±4.54^{b}	74.00±1.74^{b}
6%	72	26.83±4.83^{b}	97.96±0.41	27.92±4.60^{b}	22.46±5.16^{b}	77.38±1.45^{ab}
8%	72	28.00±5.14^{b}	98.29±0.37	24.42±3.84^{b}	26.21±5.10^{b}	75.54±1.61^{b}
20% egg yolk (C+)	72	59.75±12.83^{a}	98.04±0.56	52.21±7.96^{a}	58.04±12.36^{a}	83.46±2.99^{a}
0% + 3% (C-)	144	4.08±1.59^{b}	97.58±0.61	10.08±2.31^{b}	4.71±1.50^{b}	67.46±1.21^{c}
2%	144	3.58±1.58^{b}	97.86±0.45	17.13±4.64^{b}	3.46±1.30^{b}	76.04±2.23^{a}
4%	144	4.08±1.87^{b}	98.08±0.31	12.08±3.63^{b}	4.04±1.63^{b}	70.42±1.74^{bc}
6%	144	4.92±1.70^{b}	98.38±0.30	11.67±2.60^{b}	4.75±1.47^{b}	70.04±1.45^{bc}
8%	144	5.50±2.01^{b}	98.38±0.38	11.08±1.69^{b}	5.21±1.72^{b}	66.75±1.21^{c}
20% egg yolk (C+)	144	21.17±6.78^{a}	98.42±0.31	29.63±5.76^{a}	20.88±6.33^{a}	73.20±2.44^{ab}
P-Value	Time	0.0001	0.3962	0.0001	0.0001	0.0001
	Treatments	0.0001	0.9019	0.0001	0.0001	0.0001
	Time*Treatments	0.1480	0.9529	0.8535	0.0445	0.2422

a,b,c,d Values with different superscripts within column at the same time show a significant difference at $p < 0.05$.
VCO: virgin coconut oil; CM: coconut milk; C-: negative control (3% CM); C+: positive control (20% egg yolk)

Table 3: Effect of different concentrations of VCO combined with coconut milk in Tris-based extender on bull frozen-thawed sperm parameters at 7 and 14 days of storage (Mean ± SE).

Treatment (VCO + CM) %	**Freezing Time days**	**Parameter**				
		Motility %	**Morphology %**	**Viability %**	**Membrane integrity**	**Acrosome integrity %**
0% + 3% (C-)	7	3.42±1.70^{b}	98.29±0.42	7.63±2.90^{b}	3.83±1.39^{b}	54.42±1.74^{b}
2%	7	2.17±0.56^{b}	97.38±0.60	8.08±1.87^{b}	2.58±0.38^{b}	55.54±1.29^{b}
4%	7	2.42±0.85^{b}	98.04±0.32	10.33±3.03^{b}	2.58±0.69^{b}	55.33±1.39^{b}
6%	7	2.67±0.60^{b}	98.17±0.27	9.00±2.14^{b}	2.50±0.45^{b}	55.04±1.00^{b}
8%	7	2.67±0.53^{b}	90.33±7.77	8.67±1.94^{b}	2.42±0.40^{b}	54.92±0.93^{b}
20% egg yolk (C+)	7	46.17±3.75^{a}	98.54±0.30	42.83±5.16^{a}	45.29±4.04^{a}	80.63±1.83^{a}
0% + 3% (C-)	14	2.08±0.66^{b}	98.63±0.21	5.42±1.93^{b}	1.96±0.45^{b}	53.79±0.99^{b}
2%	14	3.33±0.89^{b}	97.88±0.30	8.75±2.37^{b}	3.17±0.72^{b}	57.63±1.78^{b}
4%	14	2.67±0.99^{b}	98.58±0.29	8.33±2.60^{b}	2.58±0.87^{b}	54.36±1.47^{b}
6%	14	3.08±1.26^{b}	98.42±0.45	7.29±2.10^{b}	3.21±1.08^{b}	54.54±1.43^{b}
8%	14	3.83±1.14^{b}	98.21±0.51	9.88±3.12^{b}	3.63±0.93^{b}	57.33±1.73^{b}
20% egg yolk (C+)	14	48.25±5.75^{a}	98.58±0.31	36.08±6.15^{a}	46.83±5.87^{a}	80.25±2.48^{a}
P-Value	Time	0.6194	0.2273	0.3344	0.7748	0.7069
	Treatments	0.0001	0.3827	0.0001	0.0001	0.0001
	Time*Treatments	0.9820	0.4688	0.8581	0.9780	0.8073

a,b Values with different superscripts within column at the same time show a significant difference at $p < 0.05$.
VCO: (virgin coconut oil); CM: coconut milk; C-: negative control (3% CM), C+: positive control (20% egg yolk).

This results supported by Abavisani *et al*. (2013) and Kandelousi *et al*. (2013) who reported supplementation of omega-3, 6, 9 fatty acids decreased the sperm motility, morphology and viability in chilled and frozen-thawed bull semen. The addition of fish oil could not improve fresh boar semen (Maldjian *et al*., 2005). However, the results in the current study do not echo those of Melo and Nunes, (1991), who showed that diluted buck semen in coconut milk gave considerable sperm motility and fertility with reasonable conception rate after insemination. Moreover, Kiernan *et al*. (2013) indicated that palmitic acid (PA), ALA and oleic acid (OA) supplemented in citrate extenders enhanced bull spermatozoa motility and viability during chilling for 7 days. According to Norman, (1962), the supplementation of coconut milk in the bovine semen extender maintained the viability of fresh semen. In the current study, the results showed that the positive control group which contained 20% egg yolk was the best values when compared with the (VCO+CM) treated groups and negative control (C-) in frozen-thawed semen. However, morphology and acrosome integrity were better value in cryopreserved semen of the (VCO+CM) treated groups compared with the others sperm parameters. These results supported by earlier study such as Amorim *et al*. (2008) who reported flaxseed oil loaded on cyclodextrin could not enhance frozen-thawed bull semen quality. Moreover, Chanapiwat *et al*. (2009) also reported no improvement by using a DHA-enriched chicken egg yolk extender on the frozen-thawed boar semen qualities. Del Valle et al. (2013) reported that addition of palm and coconut oil with the casein could not protect the sperm parameters of ram after cryopreserved like Salamon's extender including egg yolk. These findings are in contrast with Takahashi *et al*. (2012) who reported addition of palmitic acid and linoleic acid enhanced post–thawed sperm motility and viability of bovine semen. The disparity between the present study and the earlier studies such as Abavisani *et al*. (2013), Kandelousi *et al*. (2013) and Castellano *et al*. (2010) in chilled and frozen-thawed semen may be due to the types of solvent and the fatty acids used, as well as the amount of fatty acids incorporated into the extenders (Kaka *et al*., 2015b). In addition, there was an observable physical layer of the VCO during the freezing process, which may probably hinder better take-up of glycerol by the sperm. Glycerol has been found to be a good cryoprotectant that induces effective dehydration to spermatozoa at low temperature by lowering the intracellular water's freezing point (Holt, 2000). This way, the glycerol interferes with the intracellular ice crystal formation and hence protects spermatozoa from cryoinjury (Holt, 2000; Hammerstedt *et al*., 1990). Moreover, virgin coconut oil has a high percentage of saturated fatty acids (Dosumu *et al*., 2010) and solidifies at 20°C, which could clarify the difficulty of using concentrations of more than 8% in the extenders. Also, higher VCO caused a solid floating 'lid' of oil in the test tube after chilling at 5°C, due to non-emulsified oil and this finding was similar to that of del Valle *et al*. (2013).

In the current study, the different concentrations of VCO mixed with coconut milk had great benefit affect in the morphology and acrosome integrity on chilled and frozen-thawed bull semen. These results in agreement with Michael *et al*. (2007) who reported that supplementation of N-acetyl cysteine, taurine and catalase during cryopreservation expressed marked effects on the spermatozoa morphology and plasma membrane integrity. Moreover, according to Athurupana and Funahashi,

(2016), the addition of 2% coconut milk into boar semen extender significantly enhanced the acrosome integrity. Also, 0.5 mMcurcumin decreased total abnormality in cryopreserved bovine sperm (Bucak *et al*., 2012). On the other hand, addition of antioxidants did not show any marked effects on the acrosome and total abnormalities (Takahashi *et al*., 2012). This indicates that the findings of the present study due to the VCO's direct effect on the sperm through increase fluidity and flexibility of sperm plasma membrane, which increase morphology and acrosome integrity and the antioxidant properties of VCO which reduce oxidative stress (Athurupana and Funahashi, 2016). The 20% egg yolk (positive control group) compared with the different types of (VCO+CM) extenders in the present study, showed superior quality of frozen-thawed sperm, implying the inability of the (VCO+CM) to protect the sperm during cryopreservation.

Conclusion

This research was conducted to study the effect of various concentrations of VCO combined with coconut milk in Tris-based extender during chilling as well as freezing of bull sperm. VCO increased the fluidity and flexibility of sperm plasma membrane by reducing the loss of sperm lipid during cryopreservation process. Thus, our findings showed that the addition of VCO in Tris-based extender was of great beneficial effect on chilled and frozen-thawed bull semen in terms of morphology and acrosome integrity. However, Tris-based extender containing 20% egg yolk protected the quality parameters of bull sperm after chilling and freeze-thawing to a superior level than the mixtures of VCO with coconut milk.

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