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Research Article

Effect of Cholesterol and 7-Dehydrocholesterol on Bull Semen Freezing with Different Rates of Glycerol

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Abstract

The aim of this study was to evaluate the effect of 7-dehydrocholesterol loaded cyclodextrin (7-DHCLC) and cholesterol loaded cyclodextrin (CLC) with the addition of different glycerol (G) rates on bull semen cryopreservation. Semen samples of three bulls were pooled and divided into seven groups; control group (C) and the other six were extended with standard Tris extender including different G rates (3, 6 and 9%) with CLC or 7-DHCLC to reach 1.5 mg/120x10^6. Extended samples were frozen in 0.25 mL straws. After thawing, motion characteristics and motility, viability, acrosome integrity, mitochondrial activation, functional membrane integrity and abnormal spermatozoa rates were assessed. The highest membrane integrity, viability and total motility were detected in 7-DHCLC with 9% of G group (P<0.001). The lowest progressive motility was detected in 3% G groups (P<0.001). 7-DHCLC with 9% G had higher mitochondrial activity compared with 3% G groups. In addition, no statistical difference was observed between groups in terms of acrosome integrity and abnormal spermatozoa rates (P>0.05). According to these results, 7-DHCLC with 9% G addition maintained some spermatological parameters better than other groups after thawing in bull semen thus, it was concluded that 7-DHCLC could be used instead of CLC in bull semen cryopreservation.

Keywords: *Cholesterol 7-dehydrocholesterol, Cyclodextrin, Glycerol, Bull semen, Freeze-thawed*

Kolestrol ve 7-Dehidrokolestrolün Farklı Gliserol Oranları İle Boğa Sperması Dondurulmasına Etkisi

Öz

Bu çalışmanın amacı, 7-dehidrokolesterol yüklü siklodekstrin (7-DHCLC) ve kolesterol yüklü siklodekstrin (CLC) 'in farklı gliserol (G) oranları ilavesi ile boğa sperması dondurulması üzerindeki etkilerini değerlendirmektir. Üç adet boğaya ait sperma örnekleri toplandı, birleştirildi ve yedi gruba ayrıldı; biri kontrol (C) ile, diğer altı grup ise CLC veya 7-DHCLC (1.5 mg/120x10^6) ve farklı G oranlarını (%3, 6 ve 9) içeren standart Tris sulandırıcısı ile sulandırıldı. Sulandırılan spermalar 0.25 mL'lik payetlerde dondurulduu. Çözdürme sonrası gruplar hareket karakteristikleri ve motilite, viabilite, akrozom bütünlüğü, mitokondriyal aktivasyon, fonksiyonel membran bütünlüğü ve anormal spermatozoa oranları yönünden değerlendirildi. En yüksek membran bütünlüğü, viabilite ve motilite %9 G ilavesi ile 7-DHCLC grubunda tespit edildi (P<0.001). En düşük progresif motilite %3 G gruplarında saptandı (P<0.001). %9 G içeren 7-DHCLC grubu, %3 G gruplarına kıyasla daha yüksek mitokondriyal aktivite belirlendi. Ayrıca, gruplar arasında akrozom bütünlüğü ve anormal spermatozoa oranları açısından istatistiksel bir fark gözlenmedi (P>0.05). Bu sonuçlara göre, %9 G ilave edilen 7-DHCLC grubunun, diğer gruplara kıyasla eritme sonrası bazı spermatolojik parametreleri daha iyi koruduğu ve bu nedenle boğa sperması dondurulmasında CLC yerine 7-DHCLC kullanılabileceği sonucuna varıldı.

Anahtar sözcükler: *Kolesterol, 7-dehidrokolesterol, Siklodekstrin, Gliserol, Boğa sperması, Dondurma-çözdürme*

INTRODUCTION

Detrimental effects of cryopreservation on functional characteristics of spermatozoa have been well documented. During the process of semen freezing, spermatozoa have to endure a series of harmful events, including the osmotic

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pressure stress that occurs due to addition and removal of cryoprotectants [1,2], extracellular and intracellular ice formation [3], and variations in the plasma membrane lipids stimulated by shift of liquid crystalline phase to gel phase [4,5]. The most common membrane permeable cryoprotectant is glycerol (G), which restricts the ice formation within the cell [6,7]. Removal and addition of cryoprotectants, for example G, was stated to induce the osmotic damage [1,2,4]. Swelling and shrinking are responses of sperm to osmotic change that can lead to cell death and/or significant loss of membrane integrity [2].

In the female reproductive tract, the primary step of sperm capacitation is the cholesterol efflux from the plasma membrane [8]. When adequate cholesterol is removed, the membrane becomes stabilized and the acrosome membrane gains the ability to bind thus resulting in acrosome reaction [9]. Cholesterol addition to semen extender or incubation medium restricts the acrosome reaction [10,11]. On the other hand, the lipid components of spermatozoa membranes affect not only the way that sperm replies to freezing, but also the ability of sperm to capacitate and undergo the acrosome reaction [12-15]. In addition, cholesterol has a complex effect on plasma membrane property. For instance, it decreases membrane permeability and phase changes, provides a proper physical and/or chemical microenvironment for membrane proteins, regulates morphological characteristics and acts as a membrane antioxidant [16,17].

Cyclodextrins are cyclic oligosaccharides, which are the main degradation products of starch. One of the frequently used cyclodextrins, methyl-beta-cyclodextrin can solubilize hydrophobic molecules such as cholesterol. Some studies have reported that adding cholesterol loaded cyclodextrin (CLC) into bull, ram, and stallion semen extenders doubles, even triple the sperm cholesterol content [14] as well as the cholesterol-phospholipid ratio. As a result of this increase, the semen gets the least damage due to the temperature changes at the freezing stage. It is reported that addition of CLC into semen extender generally increases total motility, mitochondrial activity, membrane integrity and viability. These improvements generally observed ranging from 1-2% to 24%, mostly between 10-20% [14,18]. In addition, there are some reports indicating that treatment of sperm with CLC decreases the damage in acrosome, abnormal spermatozoa rate [19], and DNA fragmentation [20]. Additionally, Lopez-Revuelta et al.[21] stated that cholesterol modifications in cell membrane prevent the oxidation and the spreading of Reactive Oxygen Species (ROS).

In some studies demonstrated that CLC treatment with semen affect sperm membrane potential and permeability [22]. Post-thaw sperm quality positively correlated with G level and CLC. Because, CLC could be due to the changes in osmotic tolerance and permeability of the membrane to G [23]. But there have not any study about 7-DHCLC with G levels. At this points different rate of G levels were evaluated with the different cyclodextrin compounds.

7-dehydrocholesterol consists of a cholesterol upper stage (intermediate product) and cholesterol conjugates in the biochemical diagram. Therefore, it is a cholesterol conjugate, which is formed before the production of cholesterol. Amorim et al.[24] cryopreserved bull semen with the addition of cholesterol conjugates (palmitate, pelorganate, stearate and heptanoate) and obtained improved sperm quality after thawing. Likewise, Moraes et al.[25] cryopreserved bull semen with cholestenol and desmosterol, which are intermediate products of cholesterol and after thawing, the viability and motility results were increased, compared to the control group. Even though there has been only one study reported for the short-term preservation of bull semen with 7-DHCLC [26], there have not been any studies regarding bull semen cryopreservation with 7-DHCLC addition yet.

With this information taken into consideration, the aim of this study was to investigate the effect of CLC and 7-DHCLC in Tris-egg yolk extender with different G levels on bull semen cryopreservation. In addition, this study investigates the suitability of 7-DHCLC addition as a replacement for CLC since it is the first study conducted with the addition of 7-DHCLC for cryopreservation purpose of bull semen.

MATERIAL and METHODS

Cyclodextrin Preparation

Methyl-beta-cyclodextrin was loaded with cholesterol as described by Purdy and Graham [14]. A working solution of the cholesterol-loaded cyclodextrin was prepared by adding 50 mg of CLC and 7-DHCLC to 1 mL stock Tris at 37°C and mixing the solution briefly using a vortex mixer.

Animals and Semen Collection

Semen samples were collected from three Simmental bulls (2-3 years old), regularly used for breeding purpose in International Center for Livestock Research and Training (Ankara, Turkey) under uniform feeding and housing conditions. Animals were being fed with 43% barley, 10% wheat bran, 35% corn, 5% soy pulp, ad libitum salt, minerals, vitamins, dry yeast and water. A total of 30 ejaculates were collected via an artificial vagina twice a week. Following the collection, ejaculates were evaluated and only the ones with $1.0x10^9$ spermatozoa/mL sperm concentration and >80% motility were pooled and used. Pooled semen was divided into seven groups and extended in Tris egg-yolk extender (Tris 30.7 g, citric acid 16.4 g, fructose 12.6 g, 1000 mL distilled water, 20% egg yolk); one as control (C, 6% G), and the other six including different rates of G (3, 6 and 9%) with either CLC or 7-DHCLC, reaching the dose

of 1.5 mg/120x10^6. After this step, standart breeding protocol were used in this study at labarotory. Briefly, the diluted sperm samples were cooled slowly in a water bath (22°C) inside the cold cabinet and equilibrated for 4 h at 4°C [27,28]. Following the equilibration, diluted semen samples were loaded into 0.25 mL French straws and frozen in a programmable digital freezing machine (Digitcool 5300 ZB 250, IMV, France) at 3 programmed rates: -3°C/min from +4°C to -10°C, -40°C/min from -10°C to -100°C, and -20°C/min from -100°C to -140°C. Then, the straws were plunged into liquid nitrogen at -196°C.

Evaluation of Microscopic Sperm Parameters

Motility and Motion Characteristics: Computer-assisted sperm analysis (SCA, Microptics) was used to examine motion characteristics and motility. 6 µL of frozen-thawed sample was put onto a slide, mounted with a cover slide and analyzed with a 10×objective at 37°C. The total sperm motility (%), progressive motility (%), VCL (curvilinear velocity, µm s^{-1}), VSL (straight linear velocity, µm s^{-1}), VAP (average path velocity, µm s^{-1}), LIN (linearity index (LIN = (VSL/VCL) x 100), WOB (Wobble, µm), and STR (Straightness, VSL/VAP x 100) were recorded. For each evaluation, at least 250 cells in six different microscopic fields were analyzed.

Functional Membrane Integrity: The hypo-osmotic swelling test (Hos Test) was used to evaluate the integrity of the sperm membrane, based on swollen and curled tails with 100 mOsm hypoosmotic solution at 37°C for 60 min. After this period, 10 µL of the sample was put onto a slide, mounted with a cover slide and analyzed on a warm stage. Three hundred spermatozoa were evaluated under bright-field microscopy. Spermatozoa with coiled or swollen tails were recorded [29].

Abnormal Sperm Assessment: Abnormal sperm assessment was performed in accordance with sperm blue staining procedure in CASA system. Abnormal spermatozoa rate was examined by using "Sperm Blue®, Microptics®, Spain" kit [30].

Semen Viability: The kit for viable spermatozoa rate, SYBR-14/PI (Invitrogen, L-7011), was used to perform the assessment. The method previously described by Garner and Johnson [31] was used. After staining, at least two hundred sperm cells were examined under a fluorescence microscope (Leica DM 2500).

Acrosome Status: Spermatozoa acrosomal integrity was assessed using FITC-PNA (Invitrogen, L7381) and PI staining methods previously explained by Garner and Johnson [31]. After the staining, at least two hundred spermatozoa per sample were evaluated under a fluorescence microscope (Leica DM 2500).

Mitochondrial Activity: Spermatozoa mitochondrial activity was assessed using JC-1/PI (Invitrogen, T3168) staining method previously described by Garner et al.[32]. After the staining, at least two hundred spermatozoa were evaluated under a fluorescence microscope (Leica DM 2500). In the present study, the mitochondrial potential was evaluated based on total mitochondrial activity in accordance with JC-1/PI staining method. This signified that if sperm mid-piece displayed dark green to yellow/orange fluorescence, it had a mitochondrial activity, on the contrary, if it displayed pale green or there was no fluorescence in its mid-piece, the sperm had no mitochondrial activity.

All the experimental work on animals were conducted according to the regularly used for breeding purpose application by the same expert and certified veterinarians/researchers in line with the laws and regulations of the local ethical committee.

Statistical Analysis

Before performing the statistical analysis, data was examined with Shapiro-Wilk test for normality and Levene test for homogeneity of variances as parametric test assumptions. Data was subjected to one way analysis of variance (ANOVA) when the parametric test assumptions are met. Tukey test was used as post hoc procedure for significant differences. Kruskal Wallis test was used to test the difference between groups when the parametric test assumptions are violated. Dunn's multiple comparison test was used as post hoc procedure for the significant differences. A probability value of less than 0.05 was considered significant, unless otherwise noted. SPSS 14.01 was used for statistical analysis.

RESULTS

The increasing percentage of different G rates resulted in enhanced motility (56.80%) and membrane integrity (58.57%) particularly in the group 9% G with 7-DHCLC ($P<0.001$) *(Table 1)*. The lowest G concentration (3% G) caused a significant decrease in progressive motility ($P<0.001$). However, in terms of abnormal spermatozoa rate and acrosome integrity, there was no statistical significance among the groups with three different G rates ($P>0.05$). These results may refer to the membrane protective properties of CLC and 7-DHCLC. With increased G concentrations, the velocity parameters were affected positively as well, in particular, the VCL value, although interestingly, the highest STR values were obtained from the 6% G groups of CLC and 7-DHCLC ($P<0.001$) *(Table 2)*. These correlations were also confirmed by viability and mitochondrial activation values. While the viability percentage was higher in the 7-DHCLC 9% G group than control, groups with 3% G rates have shown the lowest viability ($P<0.001$) and mitochondrial activation results ($P<0.05$), indicating that a certain G threshold rate was necessary to obtain a proper amount of viable sperm after thawing *(Table 3)*.

Table 1. *Mean (±SEM) CASA motility, abnormal spermatozoa rate and membrane integrity after thawed the bull semen*

Group	Total Motility (%)	Progressive Motility (%)	Total Abnormality (%)	Hos Test (%)
CLC 3G	9.07±5.56^{c}	2.74±2.40^{c}	26.43±5.38	43.43±8.56^{bc}
CLC 6G	36.31±7.35^{b}	14.64±3.02^{ab}	25.71±3.40	47.14±7.08^{bc}
CLC 9G	36.17±9.80^{b}	11.63±4.93^{b}	28.29±9.03	53.00±4.58^{b}
7-DHCLC 3G	13.13±8.38^{c}	3.23±2.95^{c}	31.29±6.90	36.14±3.44^{c}
7-DHCLC 6G	35.93±9.23^{b}	14.99±5.08^{ab}	25.71±5.02	41.86±4.67^{c}
7-DHCLC 9G	56.80±4.34^{a}	18.31±8.78^{ab}	22.29±7.39	58.57±4.65^{a}
Control	45.33±12.33^{b}	19.84±3.39^{a}	29.71±8.62	44.29±10.48^{bc}
P	*	*	-	*

** P<0.001, Different superscripts within the same column demonstrate significant differences*

Table 2. *Mean (±SEM) CASA kinetic parameters after thawed the bull semen*

Group	VCL (μm s^{-1})	VSL (μm s^{-1})	VAP (μm s^{-1})	LIN (μm s^{-1})	STR (μm)	WOB (μm s^{-1})
CLC 3G	77.87±3.96^{c}	36.61±9.06	49.47±6.82	47.03±11.79	73.03±10.19^{ab}	63.56±8.62
CLC 6G	84.99±2.95^{b}	41.79±8.31	54.10±6.11	49.34±10.65	76.70±6.29^{a}	63.80±8.09
CLC 9G	95.11±5.67^{a}	34.11±6.13	54.09±7.38	35.76±5.28	62.87±5.11^{b}	56.70±5.12
7-DHCLC 3G	76.81±4.64^{c}	33.20±7.76	48.60±5.58	43.20±9.85	68.59±9.34^{ab}	62.34±5.81
7-DHCLC 6G	83.26±3.32^{bc}	41.20±7.52	52.47±4.96	49.80±10.36	75.70±8.08^{a}	65.09±7.49
7-DHCLC 9G	93.69±5.42^{a}	41.61±9.38	58.80±8.50	44.20±8.25	71.70±5.58^{ab}	61.23±7.09
Control	92.47±2.92^{a}	39.71±4.64	57.11±4.21	43.03±5.38	69.43±4.45^{ab}	61.81±4.67
P	*	-	-	-	*	-

** P<0.001, Different superscripts within the same column demonstrate a significant difference*

Table 3. *Mean (±SEM) viability, acrosome integrity and mitochondrial activity after thawed the bull semen*

Groups	Viability (%)	Acrosome Integrity (%)	Mitochondrial Activation (%)
CLC 3G	23.92±8.31^{d}	53.55±5.67	36.80±11.66^{b}
CLC 6G	41.02±5.67^{b}	52.27±10.02	41.38±9.80^{ab}
CLC 9G	40.52±5.19^{b}	51.25±7.33	41.59±9.43^{ab}
7-DHCLC 3G	28.52±2.87^{cd}	53.74±9.27	34.23±9.89^{b}
7-DHCLC 6G	37.05±8.60^{bc}	46.12±1.63	42.40±6.75^{ab}
7-DHCLC 9G	58.72±8.16^{a}	49.66±6.22	53.78±4.73^{a}
Control	46.83±2.87^{b}	48.32±7.87	44.94±3.62^{ab}
P	*	-	**

** P<0.001, ** P<0.05 Different superscripts within the same column demonstrate a significant difference*

DISCUSSION

In this study, cryopreservation of bull semen with the supplementation of 7-DHCLC and CLC with different rates of G were evaluated. After thawing, semen was assessed using an objective and highly technological approach in terms of sperm quality (CASA motility and motion characteristics, acrosome integrity, mitochondrial activation, viability, functional membrane integrity and abnormal spermatozoa rate).

Motamedi-Mojdehi et al.[33] observed that viability and motility were higher in all groups with different rates of CLC (1, 1.5, 3, 4.5 mg/120x10^{6}) than untreated (0 mg) groups in post-thaw ram semen. They also reported that there was no significant difference between the CLC groups (0 and 4.5 mg) in functional membrane integrity between the treated groups at different G levels (3, 5, 7%). In the present study, 1.5mg/120x10^{6} of 7-DHCLC and CLC were used with different ratios of G (3, 6, 9%) prior to freezing and after thawed HOS test was highest in 9%

G concentration. Anel et al.[34] have stated that the low level of CLC gives the possibility to decrease the required level of G for freezing. Although generally low level of G was found to preserve the volume of sperm cells and protect the cells from osmotic stress, in the current study, the highest total motility, viability and sperm membrane integrity values were observed in 9% G (high level G) group treated with 7-DHCLC when compared to other groups (P<0.001). The lowest progressive motility values were observed in 3% G (low-level G) groups both with CLC and 7-DHCLC addition (P<0.001). In addition, these results have shown that the 7-DHCLC addition has been more effective than CLC considering the cryotolerance of sperm cells, protection of their morphologic and metabolic properties from toxicity and high osmotic pressure that have been caused by G. Similar to our study, Thomas et al.[22] have demonstrated that CLC treatment affects the sperm membrane permeability. In the current study, 7-DHCLC 9% G had higher mitochondrial activation compared to both 3G groups. In accordance with our study, Blanch et al.[23] gradually increased the G concentration in CLC-treated semen, and they concluded that the post-thaw quality of sperm positively correlated with the G level. In most studies [35-37], when cryoprotectant rate is increased in the freezing media, detrimental effects induced by osmotic stress become more visible. However, the higher survival rate of sperm treated with CLC could be due to the changes in osmotic tolerance and permeability of the membrane to G [34]. In the present study, besides the cholesterol, we loaded 7-dehydrocholesterol with cyclodextrin. 7-dehydrocholesterol is a cholesterol conjugate that is formed before cholesterol is produced. Though no published data is available regarding bull semen cryopreservation with 7-DHCLC; according to our motility, membrane integrity and viability results 7-DHCLC can be used with 9% G in bull semen cryopreservation. In addition, other than VCL and STR, there was no significant difference in terms of post-thaw sperm kinetic parameters (VSL, VAP, WOB, LIN, WOB) (P>0.05). The lowest VCL values were detected in CLC and 7-DHCLC with 3% G. In bulls, the importance of VCL in fertilization capacity of spermatozoa has been well documented [31]. Therefore, these values indicate that sperm classified as highly mobile swims faster than those classified to have lower mobility [38]. Thus, the role of VCL in sperm transportation may be during the passage through the female reproductive tract and penetration to the oocyte vestments.

In several types of research, it has been demonstrated that treating spermatozoa with CLC prior to freezing increases the motility and viability post-thawed when compared to the untreated groups. Moreover, it has been stated that the addition of ≤2 mg CLC/120x10^6 has given the best results [14,18,39,40]. Although, in one study, treatment of sperm with 6 mg/mL (1.8 mg/120x10^6) CLC did not show a positive effect on sex-sorted semen after thawing, this result may be due to the harmful effect of the process on high membrane fluidity before flow cytometry sorting [39]. In a research on bull sperm with induced capacitation and acrosome reaction after thawing, *in vivo* fertilization rates were found similar in CLC-treated spermatozoa (1.5 mg CLC/120x10^6) and control group [42]. Additionally, there was no significant difference between the acrosomal integrity and abnormal spermatozoa rates of the groups. Thus, it may be asserted that cholesterol and 7-dehydrocholesterol could not decrease the percentage of morphological defects. Purdy and Graham [14] detected that when cyclic oligosaccharides of glucose with a hydrophobic center capable of incorporating lipids or cyclodextrins [43] are loaded with cholesterol and incubated with bull sperm prior to semen freezing; higher viability and motility results are observed than control groups after thawing. This cholesterol incubation may have a protective effect at low temperatures as the cells are freezing, at which the plasma membranes of spermatozoa undergo the lipid-phase transition from fluid to gel state [42]. In addition, CLC treatment could also modify the membrane permeability to penetrating cryoprotectants, since cholesterol is one of the most important regulators of membrane fluidity and permeability [44,45].

In conclusion, 7-DHCLC that has been used for the first time in bull semen cryopreservation, and better results have been obtained with the high level of G (9% G). It can be noticed that bull semen frozen with 7-DHCLC and 9% G had higher kinetic traits such as motility, progressive motility and various kinetic parameters. In addition, viability, membrane integrity and mitochondrial activity values of this group were the highest as well. According to the present study, it may be thought that membrane stabilizers such as cholesterol or its subunits could have the ability to reduce the toxic effect of G at high levels in bull semen. On the other hand, this kind of stabilization agents could allow bull spermatozoa to tolerate high osmotic pressure caused by G as well. Thus, it can be concluded that the 7-DHCLC provides protection on cellular energy metabolism, as well as structural and motion mechanism; 7-DHCLC can be used instead of CLC for bull semen cryopreservation.

Conflict to Interest

We have no *conflict of* interest to *declare.*

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