

Cryopreservation and evaluation of Akkaraman ram semen with 7-dehydrocholesterol*

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Summary: The experiment was conducted to determine the in vitro results of 7-dehydrocholesterol loaded cyclodextrin compound (7-DCLC) addition to tris extenders for freezing ram semen. Three Akkaraman rams, local species in Turkey, were used. Semen samples were extended with tris containing 0 mg (control), 1.5 mg, 2.5 mg and 3.5 mg/120x10⁶ 7-DCLC and they were equilibrated to 4°C and frozen in mini straws then stored in liquid nitrogen. Straws were thawed at 37°C for 30 s in water bath for spermatological examination. Statistically significant differences were observed between 7-DCLC and control groups in terms of acrosome integrity, CASA motility, HOS test and viability after thawing (P <0.001). In all these parameters, control group had the lowest percentages. There was no statistically significant difference between groups in terms of DNA fragmentation, abnormal spermatozoa ratio, mitochondrial activation, lipid peroxidation and apoptosis level (P> 0.05). In conclusion, it can be said that adding 7-DCLC to Akkaraman ram semen improved cell membrane cryosurvival. Besides, 7-DCLC can be used in tris extender instead of cholesterol loaded cyclodextrin (CLC) compounds for sperm cryopreservation.

Keywords: Cholesterol loaded cyclodextrin, freezing-thawing, ram sperm, 7-dehydrocholesterol loaded cyclodextrin.

Akkaraman koç spermasının 7-dehidrokolesterol ile dondurulması ve değerlendirilmesi

Özet: Bu çalışma, tris sulandırıcısına 7-dehidrokolesterol ile doyurulmuş siklodekstrin bileşimi (7-DCLC) eklenerek dondurma-çözdürme sonrası in vitro sonuçlarını değerlendirmek üzere yapıldı. Çalışmada yerli bir ırk olarak üç adet Akkaraman koç kullanıldı. Sperma örnekleri 0 mg (kontrol), 1.5, 2.5 ve 3.5 mg/120x10⁶ 7-DCLC içeren tris sulandırıcısı ile sulandırıldı, 4 C°'de alışıma bırakıldı ve mini payetlerde dondurularak sıvı azotta saklandı. Daha sonra payetler spermatolojik analizler için 37 C°'de 30 saniyede su banyosunda çözdürüldü. Çözüm sonu, 7-DCLC grupları ve kontrol grubu arasında CASA motilitesi, canlılık, akrozomal bütünlük ve HOS test yönünden istatistiksel olarak farklılık önemli bulundu (P<0.001). Bütün bu parametrelerde kontrol gruplarında en düşük değerler tespit edildi. Anormal spermatozoa oranı, lipit peroksidasyon seviyesi, DNA hasarı, mitokondriyal aktivasyon, lipit peroksidasyon ve apoptozis seviyesi yönünden istatistiksel açıdan bir farklılık bulunamadı (P>0.05). Çalışma sonucunda, 7-DCLC'nin Akkaraman koç spermasına eklenmesinin hücre membranlarını soğuktan koruduğu söylenebilir. Ayrıca, spermanın dondurulması için tris sulandırıcılarında kolesterol ile doyurulmuş siklodekstrin (CLC) bileşimi yerine 7-DCLC'nin kullanılabileceği tespit edildi.

Anahtar sözcükler: Çözdürme, dondurma, koç sperması, kolesterol ile doyurulmuş siklodekstrin, 7-dehidrokolesterol ile doyurulmuş siklodekstrin.

Introduction

Ram spermatozoa has a special composition of cell membrane that makes difficult to cryopreserve well (1). Ram sperm has a lower phospholipid / cholesterol ratio and a higher fatty acids / polyunsaturated ratio than other species (11). Sperm susceptibility to cryopreservation is explained by membrane phospholipid ratio and membrane cholesterol to phospholipid composition (15). Besides, changing lipid structure of sperm membrane affects sperm freezing (2). It means that sperms having low cholesterol: phospholipid rates from boars, stallions, rams and bulls are more sensitive to the cryo damage than sperms from

rabbit and human which have high cholesterol: phospholipid ratios (31, 47).

During the last twenty years, authors have been working about sperm membrane with CLC, aiming to protect sperm from cold injury in different animals (25). Cholesterol is a necessary sterol for cell proliferation and viability. In addition, cholesterol enables cell interactions, affects membrane phase transition, ensures suitable environments for membrane-associated proteins, facilitates morphological membrane characteristics, serves as a membrane antioxidant and decreases membrane permeability by stabilizing the membrane (9).

* Bu çalışma "Akkaraman koçlarda spermanın kolesterol ve 7-dehidrokolesterol ile dondurulması ve değerlendirilmesi" adlı tezden üretilmiştir.

Cyclodextrins are cyclic oligosaccharides which are main degradation yields of starch. Methyl beta cyclodextrin, which is one of the most used cyclodextrin, can solubilise hydrophobic molecules such as cholesterol. Recently, the using of CLC was proposed to facilitate cholesterol transfer into the plasma membranes of bull spermatozoa (33). 7-dehydrocholesterol is a cholesterol conjugating and consisting of cholesterol upper stage (intermediate product) in biochemical diagram meaning that it is one of the cholesterol conjugates and 7-DCLC is formed before cholesterol is produced. Moraes et al. (23) cryopreserved bull semen with cholesterol and desmosterol, which are intermediate products of cholesterol and the motility and viability results were increased compared to the control group after thawing. Amorim et al. (2) cryopreserved bull semen with cholesterol conjugates (heptanoate, palmitate, pelorganate and stearate) and obtained increased percentages of sperm parameters after thawing. However, generally there are no studies demonstrating cryopreservation with 7-DCLC in any animal species.

With the light of these informations, the aim of this study was to discover the protective effects of different doses of 7-DCLC added on tris-egg yolk extender against freezing and thawing procedure (cryo-damage).

Materials and Methods

Chemicals: All chemicals used in this study were obtained from Sigma Chemical Company (St. Louis, MO, USA).

Cyclodextrin preparation: Methyl beta cyclodextrin was loaded with 7-dehydrocholesterol as explained by Purdy and Graham (33).

Animals: Semen samples from three Akkaraman rams (2 and 3 years of ages) were used in the present study. The rams were kept in uniform breeding conditions at the International Livestock Research and Education Center in Lalahan - Ankara. A total number of 30 ejaculates were taken from the rams with the aid of artificial vagina and the semen were pooled to decrease individual variation during the breeding season. Spermatological examination was performed within 10 minutes after taking the semen. The study was approved by the Animal Care Committee of Ankara University (2013-10-72).

Semen processing: A Tris extender with 10% egg yolk and 5% glycerol were used. The good quality ejaculates (volume ≥ 0.5 mL, mass activity: ≥ 4 ; motility: $\geq 80\%$, sperm concentration: $\geq 2 \times 10^9$ /mL) were pooled. Each pooled ejaculate was split in to four groups, one has no additives (control), three of these were received 7-DCLC (1.5 mg, 2.5 mg, 3.5 mg/ 120×10^6) and final concentration was 500×10^6 spermatozoa/ml. Samples were cooled slowly in the water bath (22°C) inside the cold cabine (4°C) and equilibrated for 4 hours. Diluted

samples were filled in mini straws (0.25 ml) after equilibration, then samples were frozen with a digital freezing machine (IMV, France). After being stored for at least 24 hours, straws were thawed in a water bath for spermatological examination.

Evaluation of spermatological parameters:

CASA motility: Computer aided sperm analyse (SCA®) was used for the examination of motility and kinetic parameters. The spermatozoa motility properties were set as static, slow $> 60 \mu\text{m/s}$, medium $> 90 \mu\text{m/s}$, fast $> 120 \mu\text{m/s}$. Six μl drop of thawed semen was put onto slide and covered with a coverslide and then motilities were analysed with a 10 x objective at 37 °C. The following motilities were recorded: progressive motility (%), total sperm motility (%), VSL ($\mu\text{m s}^{-1}$), VAP ($\mu\text{m s}^{-1}$), WOB (μm), VCL ($\mu\text{m s}^{-1}$), LIN (VSL/VCL x 100) and STR (VSL/VAP x 100) For each evaluation, at least 200 and most 300 sperm cells were analysed in six microscopic fields (47).

HOS test: The hypoosmotic swelling test (HOS test) was used to examine the integrity of the sperm membrane, based on swollen tails and was performed by incubating 100 mOsm hypoosmotic solution at 37°C for 60 minutes. Two hundred sperm cells were evaluated under bright-field microscopy. Sperm with swollen or coiled tails were recorded (44).

Abnormal sperm assessment: Analysis of abnormal sperm assessment was performed with sperm blue staining procedure in CASA system. Examination of abnormal spermatozoa ratio was done with “Sperm Blue®, Microoptics®, Spain” kit (45).

Dead spermatozoa rate (viability): The percentage of dead spermatozoa was determined by sperm viability kit (SYBR-14 / PI, Invitrogen, L 7011), assessing the feasibility of the method which was modified from the study of Garner & Johnson (13), previously described by Bucak (8). After staining, at least two hundred sperm cells from each specimen were analysed with fluorescent microscope (Leica DM 3000).

Acrosome status: Sperm acrosome status was done with FITC-PNA (Invitrogen, L7381) and PI by staining method, previously explained by Bucak et al. (8) and modified from the study of Nagy et al. (24). After staining, at least two hundred sperm cells were examined with a fluorescent microscope (Leica DM 3000).

Mitochondrial activity: Sperm mitochondrial activity was done with JC-1/PI (Invitrogen, T3168) by staining method previously described by Cayan et al. (11) and modified from a study of Garner & Johnson (13). After staining, at least two hundred sperm cells were examined with a fluorescent microscope (Leica DM 3000).

DNA damage: The sperm DNA damage was examined by using the single cell gel electrophoresis (comet) assay previously explained by Tuncer et al. (43).

After analysis, one hundred nuclei were examined using a fluorescent microscope (Zeiss, Germany).

Lipid peroxidation levels: The lipid peroxidation (LPO) and total antioxidant level (tGSH) were examined by the previously described methods by Baspınar et al. (5); antioxidant activity (AOP) was examined according to Bucak et al. (8) method by spectrophotometry.

Detection of apoptosis-like changes: Spermatozoon apoptosis was investigated using Annexin V-PI assay (phosphotidil-serine translocation). After thawing, samples were centrifugated at 300 G for 10 minutes. The semen were washed twice with 1x binding buffer to 1×10^6 spermatozoa/cm³ for removing the seminal plasma. Then 5 µl Annexin V and 5 µl PI were added to samples and incubated 15 minutes in a moist box at room temperature. Afterwards, samples were read with FACS calibur™ flow cytometry and apoptotic cell analyzes were done by BD Cell Quest™ software programme. After the examination of semen; early apoptotic and late apoptotic spermatozoon rates were recorded.

Statistical analysis: The significant difference among the groups was analysed by ANOVA. To determine differences between the groups, Tukey test was used. Besides, statistical analyses were done with a minimum 5% margin of error. The descriptive measurements of groups were given in the tables as “Arithmetic Mean (\bar{X}) ± Standard Deviation (SD)”.

SPSS® for Windows 14.1 (Licence No:9869264) package programme was used for the analysis of the data.

Results

As shown in Table 1, 7-DCLC groups yielded higher percentages of total motility than control after thawing ($P < 0.001$). But there were no differences among the groups in progressive motility ($P > 0.05$). Likewise, the 7-DCLC addition did not improve the sperm motility kinetic parameters (VCL, VSL, VAP, LIN, STR, and WOB). Control group showed the lowest HOS test ($P < 0.001$). Besides, there was no significant difference in the abnormal spermatozoa rate among the groups ($P > 0.05$).

As presented in Table 2, there was no significant difference in DNA damage among the treatment groups ($P > 0.05$).

As shown in Table 3, 7-DCLC groups produced a higher viability and acrosomal integrity compared to the control group ($P < 0.001$). However, there was no significant difference among the groups in terms of mitochondrial activity ($P > 0.05$).

As shown in Table 4, there was no significant difference in LPO, AOP and tGSH levels ($P > 0.05$).

As shown in Table 5, there was no significant difference in apoptosis parameters (early apoptotic and late apoptotic cells) ($P > 0.05$).

Table 1. Mean (\pm SD) CASA motility, kinetic parameters, HOS test and total abnormal spermatozoa rate values after thawing the semen. Tablo 1. Çözüm sonu ortalama CASA motilitesi, kinetik parametre, HOS test ve total anormal spermatozoa oranı değerleri.

Analysis	1.5 mg	2.5 mg	3.5 mg	Control	P
Casa Motility (%)	54.23 \pm 0.09 ^a	54.00 \pm 0.07 ^a	56.00 \pm 0.06 ^a	33.00 \pm 0.09 ^b	***
Progressive Motility (%)	13.00 \pm 0.05	12.00 \pm 0.03	13.00 \pm 0.04	8.00 \pm 0.04	-
VCL (µm/s)	97.98 \pm 9.73	97.07 \pm 6.62	95.98 \pm 6.14	94.19 \pm 3.10	-
VSL (µm/s)	49.43 \pm 3.47	50.91 \pm 2.74	51.63 \pm 9.79	49.73 \pm 8.79	-
VAP (µm/s)	71.42 \pm 8.79	71.99 \pm 5.29	71.12 \pm 8.92	66.93 \pm 9.56	-
LIN (%)	53.23 \pm 5.92	53.87 \pm 2.81	53.66 \pm 8.53	50.63 \pm 4.49	-
STR (%)	72.88 \pm 3.74	72.61 \pm 2.31	72.09 \pm 4.89	74.01 \pm 2.77	-
WOB (%)	72.89 \pm 4.56	74.16 \pm 1.55	74.03 \pm 7.03	68.36 \pm 4.17	-
HOS(%)	56.78 \pm 7.61 ^a	56.00 \pm 15.20 ^a	58.22 \pm 11.20 ^a	36.44 \pm 18.20 ^b	***
Total Abnormalities (%)	18.00 \pm 0.08	20.00 \pm 0.05	17.00 \pm 0.08	20.00 \pm 0.07	-

^{a,b}: Different superscripts within the same row demonstrate significant differences ($P < 0.001$).

- : No significant difference ($P > 0.05$).

^{a,b}: Aynı satırda farklı harfleri taşıyan değerler istatistiksel açıdan önemlidir ($P < 0.001$).

- : İstatistiksel olarak bir fark yoktur ($P > 0.05$).

Table 2. Mean (\pm SD) DNA damage values after thawing the semen.

Tablo 2. Çözüm sonu ortalama DNA hasarı değerleri.

Analysis	1.5 mg	2.5 mg	3.5 mg	Control	P
Tail length	54.98 \pm 22.57	54.21 \pm 23.65	51.89 \pm 21.72	60.31 \pm 23.57	-
Tail intensity (%)	11.52 \pm 2.13	11.41 \pm 2.29	10.25 \pm 2.47	11.84 \pm 3.00	-
Tail moment (µm/s)	3.18 \pm 1.72	3.00 \pm 1.80	2.79 \pm 1.57	3.61 \pm 2.94	-

- : No significant difference ($P > 0.05$).

- : İstatistiksel olarak bir fark yoktur ($P > 0.05$).

Table 3. Mean (\pm SD) fluorescent staining parameters after thawing the semen.

Tablo 3. Çözüm sonu ortalama floresan boyama değerleri.

Analysis	1.5 mg	2.5 mg	3.5 mg	Control	P
Sperm viability (%)	56.68 \pm 11.64 ^a	55.21 \pm 6.61 ^a	58.07 \pm 6.63 ^a	35.03 \pm 9.64 ^b	***
Acrosome integrity (%)	70.76 \pm 13.4 ^a	80.30 \pm 6.27 ^a	76.10 \pm 8.60 ^a	57.49 \pm 10.8 ^b	***
Mitochondrial activity (%)	77.9 \pm 11.04	71.9 \pm 12.07	69.9 \pm 16.03	61.9 \pm 21.08	-

^{a,b}: Different superscripts within the same row demonstrate significant differences ($P < 0.001$).

- : No significant difference ($P > 0.05$).

^{a,b}: Aynı satırda farklı harfleri taşıyan değerler istatistiksel açıdan önemlidir ($P < 0.001$).

- : İstatistiksel olarak bir fark yoktur ($P > 0.05$).

Table 4. Mean (\pm SD) LPO levels ($\mu\text{M} \times 10^9$), AOP ($\text{mM} \times 10^9$) and tGSH ($\mu\text{M}/1 \times 10^9$ sp) after thawing the semen.Tablo 4. Çözüm sonu ortalama LPO ($\mu\text{M} \times 10^9$), AOP ($\text{mM} \times 10^9$) ve tGSH ($\mu\text{M}/1 \times 10^9$ sp) değerleri.

Analysis	1.5 mg	2.5 mg	3.5 mg	Control	P
LPO ($\mu\text{M} \times 10^9$)	20.37 \pm 1.18	20.98 \pm 1.27	26.66 \pm 2.97	23.72 \pm 2.57	-
AOP ($\text{mM} \times 10^9$)	12.0 \pm 0.76	13.0 \pm 0.94	13.0 \pm 0.32	14.0 \pm 1.57	-
tGSH ($\mu\text{M} 1 \times 10^9$)	945 \pm 283.8	758 \pm 144.7	718 \pm 214.9	861 \pm 357.7	-

- : No significant difference ($P > 0.05$).

- : İstatistiksel olarak bir fark yoktur ($P > 0.05$).

Table 5. Mean (\pm SD) apoptosis parameters after thawing the semen.

Tablo 5. Çözüm sonu ortalama apoptosis değerleri.

Analysis	1.5 mg	2.5 mg	3.5 mg	Control	P
Early Apoptotic (%)	9.52 \pm 4.61	10.48 \pm 4.77	11.06 \pm 3.50	11.67 \pm 4.99	-
Late Apoptotic (%)	90.46 \pm 1.48	88.92 \pm 1.62	88.92 \pm 1.23	87.89 \pm 1.72	-

- : No significant difference ($P > 0.05$).

- : İstatistiksel olarak bir fark yoktur ($P > 0.05$).

Discussion and Conclusion

This study was evaluated the effect of 7-DCLC on freezed-thawed ram semen. In this study, 7-DCLC increased percentages of progressive and total motility after thawing. Similar results were found in other cholesterol conjugates (2, 23). Also some authors found improvement both in motility and sperm membrane integrity after incubating the semen with CLC prior the cryopreservation (3, 21, 34). Although we did not find any statistically significant difference ($P > 0.05$) in terms of post thaw sperm kinetic parameters, 7-DCLC had numerically positive effect on linear movement direction and velocity. Oliveira et al. (29) reported similar results about the kinetic parameters.

The HOS test determines the resistance of the sperm plasma membrane to stimulate by the damage of permeability in the stress of hypoosmotic fluid (6). In this study, the highest HOS test rates were found in 7-DCLC at 1.5, 2.5, 3.5 mg/120 \times 10⁶. In the present study we found a positive relation among total motility, progressive motility, viability and HOS test. In terms of both motility and HOS test compared to control group lower percentages were obtained in the present study ($P < 0.001$). These relations were seen in accordance with other authors reports (4, 20, 26, 39). Because these spermatological

parameters (motility, HOS test, viability) are dependent on sperm plasma membrane integrity, similar results are expected and their results confirm our results. Although some authors (9, 20) found correlation between morphological defect and HOS test, we did not observe any interaction between these parameters. So, these parameters should be evaluated separately.

Mitochondria are located in the sperm mid-piece and they produce accessible energy to the tail filaments, so facilitate effect of the sperm both to reach the oocyte and penetrate to zona pellucida (10). There are dense fibers located to axonem in sperm cell mid-piece (mitochondria). These fibers provide potential source to produce ATP energy and spermatozoa motility (12). Mitochondrial potential changes could be a good sign of physiological disruption (7). Some authors, who worked with different CLC levels, found higher mitochondrial activity than control groups (36, 37, 38). Although there was no statistically significant difference among the groups, 7-DCLC groups had higher percentages of mitochondrial activity than the control group in the present study.

The COMET method is a widely used assay for analyzing DNA breakage in individual cells (30, 35). It is also very good method to evaluate the maintainance of genetic material integrity in biological studies (27, 42).

There was no significant difference between 7-DCLC groups and the control group in terms of the maintenance of DNA integrity in this study. These results are consistent with studies on boar (41) and human with infertility problems (17). However, Katanbegzadeh et al. (18) suggested that CLC had a positive effect on DNA fragmentation. Hence, DNA structure evaluation of sperm might be a useful tool for accurate prediction of the male fertility in individual rams.

In the present study samples were evaluated by flow cytometry (Annexin V-PI) for apoptosis. Although there was no statistically significant difference among the groups for apoptosis parameters (early apoptotic and late apoptotic cells) ($P>0.05$); high apoptotic level was obtained in the whole groups. The cryopreservation process acts as an apoptotic effect in ram semen (28). This suggestion and our results are agreed with other results reported (16, 22, 32). This might have occurred as a result of our freezing-thawing procedure. Cryopreservation causes a number of damages in sperm cells induced by different causes such as osmotic and toxic stresses, cold shock and ice in the environment and semen extender type or animals (34, 46). Another important reason might be the supplementation of cholesterol conjugate (7-DCLC). Inoue et al. (16) hypothesized that, cholestanol which is a cholesterol conjugate, might induce neuronal cell death in the cerebellum and lead to cerebellar ataxia in serum of Cerebrotendinous Xanthomatosis (CTX) patients. To support this hypothesis, hyper-cholestanolemic rats were created in that study by feeding cholestanol. Similar to the results, our 7-DCLC might have had a negative effect for apoptosis. However, this hypothesis needs to be supported by further research and in vivo fertility (artificial insemination).

This study was performed to investigate that 7-DCLC would prevent oxidation defects during the freezing of ram semen. There was no statistically significant difference among the groups for oxidative parameters (LPO, AOP and tGSH) ($P>0.05$). These findings of lipid peroxidation level and antioxidant capacity were the same with other authors findings for ram semen (14, 25). However, Lopez-Revelta et al. (19) reported that cholesterol modification in the cell membrane protects antioxidative enzymes and prevents spreading to radio oxidative species (ROS).

In conclusion, 7-DCLC can improve motility, viability, plasma membrane integrity and acrosome integrity. 7-DCLC did not improve mitochondrial activation, DNA damage, abnormal spermatozoa rate, apoptosis, LOP, AOP and tGSH levels in ram semen. Therefore it can be said that adding 7-DCLC to ram sperm membrane improved cell cryosurvival and 7-DCLC can be used in tris extender instead of cholesterol loaded cyclodextrin (CLC).

Acknowledgements

We would like to thank the Scientific and Technological Research Council of Turkey (TUBITAK) for financially supporting this study (project no: 113O775).

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Geliş tarihi: 05.12.2016 / Kabul tarihi: 21.03.2017

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