Kocatepe Veterinary Journal

Kocatepe Vet J. (2023):16(3):439-443

DOI: 10.30607/kvj. 1347383

RESEARCH ARTICLE

Effects of Insulin Lispro on Ram Semen During Cryopreservation

Nail Tekin ÖNDER[1]*, Taygun GÖKDEMİR[1], Muhammet Can KILIÇ[1], Savaş YILDIZ[1], Yavuz ÖZTÜRKLER[1]

[1]*Department of Artificial Insemination, Faculty of Veterinary Medicine, Kafkas University, Kars, Türkiye*

ABSTRACT

In the present study, it was aimed to evaluate the effects of semen extender supplemented with insulin on ram semen at the post-thaw stage. Semen was collected from 5 Tuj rams five times with an electro-ejaculator. Experimental groups were designated as control group and groups containing 15 IU and 20 IU of insulin lispro supplemented semen extenders. At the post-thaw stage, motility and plasma membrane integrity were evaluated by microscopy. Acrosome integrity (Fitc-Peanut Agglutinin) and mitochondrial membrane potential (Rhodamine 123) were assessed by dual staining with propidium iodide (PI) using flow cytometry. It was found that motility and plasma membrane functional integrity were better preserved in the experimental groups than the control group ($p<0.05$). Acrosome integrity results were similar between the control and 15 IU insulin groups ($p>0.05$), but acrosome integrity was negatively affected in the 20 IU insulin group ($p<0.05$). Compared to the control, mitochondrial membrane potential was found to be higher in the group containing 20 IU insulin ($p<0.05$). As a result, it was thought that energy metabolism was stimulated in ram semen frozen with insulin-supplemented extenders, and the semen was better preserved than the control group.

Keywords: Cryopreservation, insulin, ram semen, tuj

***

Insülin Lispro'nun Kriyoprezervasyon Sırasında Koç Sperması Üzerine Etkileri

ÖZ

Sunulan çalışmada, insülin ilave edilen sperma sulandırıcısının koç sperması üzerindeki etkilerininin çözdürme sonrası aşamada değerlendirilmesi amaçlanmıştır. Tuj ırkı 5 adet koçtan beş kez elektro-ejakülatör ile sperma alındı. Deneme grupları, kontrol grubu, 15 IU ve 20 IU insulin lispro ilave edilen sperma sulandırıcıları ile oluşturuldu. Çözdürme sonrası, motilite ve plazma membran bütünlüğü mikroskopi ile değerlendirildi. Akrozom bütünlüğü (Fitc-Peanut Agglutinin) ve mitokondrial membran potansiyeli (Rhodamine 123) akış sitometrisi kullanılarak, propidium iodide (PI) ile ikili boyama yapılarak değerlendirildi. Motilite ve plazma membran fonksiyonel bütünlüğünün, araştırma gruplarında, kontrol grubuna göre daha iyi korunduğu bulundu ($p<0,05$). Akrozom bütünlüğü sonuçlarının kontrol ve 15 IU insülin grupları arasında benzer olduğu ($p>0.05$), ancak 20 IU insulin grubunda akrozom bütünlüğünün olumsuz etkilediği bulundu ($p<0,05$). Mitokondrial membran potansiyeli, 20 IU insulin içeren grupta, kontrol grubuna göre daha yüksek bulundu ($p<0,05$). Sonuç olarak, insulin ilave edilmiş sulandırıcılar ile dondurulan koç spermasında enerji metabolizmasının uyarıldığı ve spermanın kontrol grubuna göre daha iyi korunduğu düşünülmüştür.

Anahtar kelimeler: İnsülin, koç sperması, kriyoprezervasyon, tuj

To cite this article: Önder N.T. Gökdemir T. Kılıç M.C. Yıldız S. Öztürkler Y. Effects of Insulin Lispro on Ram Semen During Cryopreservation. Kocatepe Vet J. (2023):16(3):439-443

Submission: 16.06.2023 **Accepted**: 25.09.2023 **Published Online**: 27.09.2023

ORCID ID; *NTÖ: 0000-0001-5141-0008, TG: 0000-0001-6049-7184, MCK: 0000-0003-0771-3970, SY: 0000-0001-6459-6841, YÖ: 0000-0002-0681-9311*

*Corresponding author e-mail: nailtekinonder@gmail.com

INTRODUCTION

Beta cells located in the Islets of Langerhans within the pancreas are responsible for the production of insulin. The hormone insulin, comprises 51 amino acids and possessing a double-chain polypeptide structure, was first isolated by Banting and Best in 1922 (Karamitsos 2011, Koyunlu 2023). The hormone insulin, which plays a crucial role in energy metabolism, also has a role in the regulation of reproductive functions (Dias et al. 2014). The disease known as diabetes mellitus, which is characterized by elevated levels of blood glucose, has been found to have a negative impact on the reproductive functions of males (Dias et al. 2014, Lunze et al. 2013). The negative impact of spermatological parameters, such as decreased motility and morphological disorders in diabetes mellitus can lead to a reduction in male fertility (Dias et al. 2014).

Insulin has been added into semen extenders for ram, human, and pig semen due to its potential involvement in the energy metabolism of spermatozoa. This inclusion is believed to enhance motility and reduce observed morphological disorders (Aquilla et al. 2013, Lampiao and du Plessis 2008, van Tilburg et al. 2021). There is an investigation conducted on the semen of rams focused on the assessment of short term semen preservation (van Tilburg et al. 2021), while the impact of such preservation on the subsequent freezing-thawing procedures was not taken into account. Insulin lispro, an insulin analogue, has a rapid onset of action and a shorter duration compared to endogenous insulin and is administered to humans by subcutaneous injection. According to reports, the peak level is achieved between 30 and 90 minutes after subcutaneous injection, and the duration of the action ends in under 5 hours (Noble et al. 1998). Furthermore, there have been reports indicating that it exhibits comparable sensitivity to insulin receptors, similar to the effects of regular insulin (Holleman and Hoekstra 1997). Insulin lispro was chosen as the optimal treatment strategy in the study due to the fact that the duration between semen dilution and freezing was much less than 5 hours. The current investigation aims to examine the impact of insulin lispro-supplemented semen extender on the reconstitution and cryopreservation of ram spermatozoa.

MATERIAL and METHODS

All issues associated to the experimental setups and evaluation techniques have been approved by The Scientific Ethical Committee at the Kafkas University in Kars, Türkiye (2021–196). Five different Tuj rams of the 3-5 years old were used and maintained at Prof. Dr. Ali Riza Aksoy Education, Research and Application Farm, Kafkas University, Faculty of Veterinary Medicine in Kars, Türkiye.

Chemicals

Except where otherwise noted, all of the chemicals used in the study were purchased from Merck and Sigma.

Experimental Design

The research was designed to include three distinct groups, namely those that received 15 IU and 20 IU of insulin lispro (Humalog, Lilly, Italy) in the extenders, and the control group without insulin lispro. Insulin doses of 15 IU (I-15) and 20 IU (I-20) were administered on a 3 mL of extenders. The extender was composed of 223.7 mM Tris, 55.5 mM fructose, 66.6 mM citric acid, 100.4 mM Trehalose, 4.03 mM EDTA, 4 g/l penicillin G, 3 g/L dihydrostreptomycin, and 20% egg yolk (v/v) in distilled water.

The process of semen collection was conducted five times, with a frequency of every other day, utilizing an electro-ejaculator. Following the collection process, the ejaculates were subsequently transferred to a water bath maintained at a temperature of 37°C. Using a heated (37°C) slide, a phase-contrast microscope (Nikon Eclipse-E400, Tokyo, Japan) was used to assess the rapid wave motion and motility. Semen samples were pooled and exhibiting a motility rate exceeding 70% and containing over 1.5x109 spermatozoon/ml were selected for the purpose of cryopreservation.

The dilution process involved reducing the concentration of each group to 25x106 sperm/ml using a corresponding extender. The groups were subsequently brought down to a temperature of 5°C within a time frame of one hour. After undergoing the cooling process, the sperm samples were allowed to reach equilibrium for a duration of two hours at a temperature of 5°C. The techniques employed for cryopreservation, thawing, and incubation were executed in accordance with the methodology outlined by Yildiz et al. (2015).

Semen Analysis

The subjective evaluation of sperm motility was conducted using a 400x phase-contrast microscope, with a slide warmed to 37°C. The functional integrity of the plasma membrane was assessed through the hypoosmotic swelling test (HOST), as per the method described by Alcay et al. (2016).

Flow Cytometric Analysis

The Attune NxT Acoustic Focusing Cytometer (Invitrogen, USA) was used to conduct flow cytometric analysis. The fluorescence was assessed using a 480 nm excitation wavelength with a 10 nm excitation bandwidth. The emission was filtered using 530/30 nm filter (BL-1) and 695/40 nm filter (BL-3) and connected to Attune NxT software v2.7 (Thermo

Fisher). Upon gating the cell population based on forward and side scatter light signals, the mean fluorescence intensity of the analyzed sperm cells was quantified. The assay contained a quantity of 10,000 sperm cells.

The evaluation of acrosome integrity was conducted using the dual-staining technique of fluorescein isothiocyanate-conjugated peanut agglutinin (PNA) and propidium iodide (PI). The evaluation of mitochondrial membrane potential was carried out using Rhodamine 123/PI. The flow cytometric analysis was conducted following the methodology outlined by Gürler et al. (2016).

Statistical Analysis

Statistical analysis was conducted using IBM SPSS version 28. The normality of the data was evaluated using the Shapiro-Wilk test. The presented information was displayed in the form of mean values accompanied by their corresponding standard errors. The statistical significance of inter-group differences was assessed through the utilization of one-way ANOVA, followed by Tukey's post-hoc test. The statistical analysis employed to investigate data with a non-normal distribution was the Kruskal-Wallis test. Statistical significance was determined for P values that were less than 0.05.

RESULTS

Table 1 provides an extensive overview of motility, plasma membrane functional integrity, acrosome integrity and mitochondrial membrane potential concentration.

Post cryopreservation, the motility percentages were recorded as 44.44%, 50.56%, and 48.33% in the control, I-15, and I-20 groups, respectively. The preservation of motility was observed to be significantly better in groups that were administered with insulin ($P<0.05$).

HOST results of the study were found as 65.00% in control group, 73.22% in I-15 and 70.44% in I-20. The study revealed that semen dilution that included insulin exhibited a superior ability to maintain the integrity of the plasma membrane in comparison to the control group (C) ($P<0.05$).

The study found that there was no significant difference in the total acrosome integrity (C: 79.12%; I-15: 80.91) and the acrosome integrity with PI staining (C: 53.90%; I-15: 53.91%) ratio between the control group and the I-15 group ($P>0.05$). However, the I-20 group had significantly lower acrosome integrity (I-20: 74.67% in PNA and 46.53% in PNA/PI) compared to the other groups ($P<0.05$). The results of the analysis indicate that the mitochondrial membrane potential was found to be significantly higher in I-20 compared to control group (C: 74.08%; I-20: 79.35%) ($P<0.05$). Mitochondrial membrane potential results in Rhodamine 123/PI were found to be similar in all groups ($P>0.05$).

Table 1. Effect of insulin on spermatological parameters

Measurements	Motility (%)	HOST (%)	A (%)	A-P (%)	M (%)	M-P (%)
Groups						
Control	44,44±1,30^{a}	65,00±1,09^{a}	79,12±0,92^{a}	53,90±1,90^{a}	74,08±1,42^{a}	37.84±1.64
I-15	50,56±1,00^{b}	73,22±1,05^{b}	80,91±0,64^{a}	53,91±1,53^{a}	78,41±1,17^{ab}	38.21±2.25
I-20	48,33±0,83^{b}	70,44±0,88^{b}	74,67±1,21^{b}	46,53±1,20^{b}	79,35±1,44^{b}	38.44±1.79

^{a,b}: Values with different superscripts in the same column for each times are significantly different ($P < 0.05$). HOST: Plasma Membrane Functional Integrity, A: Total Acrosome Integrity, A-P: Acrosome integrity with Intact Plasma Membrane, M: Total Mitochondrial Membrane Potential, M-P: Mitochondrial Membrane Potential with Intact Plasma Membrane.

DISCUSSION

The freezing of gamete cells has a number of detrimental impacts on cells. In order to lessen negative effects like reduced viability and motility in semen as well as morphological problems, experiments have been conducted on adding antioxidant substances or other sources to semen extenders (Alcay et al. 2016, Alcay et al. 2021, Alcay et al. 2022). Insulin has a key role in spermatogenesis and spermatozoa's metabolic activity (Aitken et al. 2021, Bruning et al. 2000). Low insulin levels in the blood plasma have a negative impact on spermatological parameters in bulls (Weerakoon et al. 2020). The present study aimed to investigate the effects of insulin supplementation on ram semen extenders after cryopreservation, given the crucial role of insulin in reproductive performance. The assessment of motility is an important component in numerous research endeavors, as the forward movement of spermatozoa within the female reproductive tract is a critical factor in achieving successful fertilization. Studies have reported a positive impact on motility when substances involved in energy metabolism are added into sperm extenders (Aquilla et al. 2013, Lampiao and du Plessis 2008,

Onder et al. 2022). The findings of our research, in conjunction with other studies, demonstrated a significant enhancement in the motility of ram sperm. The findings of van Tilburg et al.'s (2021) study, which involved the addition of insulin to the ram's semen extender during short-term storage, align with the results of our own investigation, as we also observed a positive impact on the motility.

The process of freezing sperm has been found to result in damage to the membrane structure of the spermatozoa, ultimately leading to a decrease in the efficacy of the freezing process. The researchers assess the integrity of the membrane subsequent to the process of freezing and thawing, as the adverse impacts on the structure of the membrane can lead to a reduction in potential fertility (Onder et al. 2022, Vazquez et al. 1997). Based on the results of the HOS test, our study revealed that the insulin-containing groups exhibited a higher rate of membrane integrity. The study conducted by van Tilburg et al. (2021), reported the absence of any noticeable variation in the integrity of the plasma membrane. The dissimilarity noted between the current investigation and the aforementioned study could potentially be attributed to the administration of elevated insulin dosages in our study.

According to reports, the inclusion of insulin in extenders has been found to induce the acrosome reaction in spermatozoa (Lampiao and du Plessis 2008, Silvestroni et al. 1992). The findings of our investigation indicate that the reduction in overall acrosome integrity and acrosome integrity in viable cells at elevated insulin levels aligns with the existing knowledge on this subject. Another study's finding indicated that the addition of insulin during the short-term storage of ram semen did not result in any significant alteration in acrosome integrity (van Tilburg et al. 2021). The researchers in the previously mentioned study have indicated that there may be a correlation between dosage and the stimulation of the acrosome reaction. Specifically, high doses may potentially stimulate this reaction. The results obtained in our research validate the notion suggested by the preceding study.

For the sustenance of spermatozoa motility, it is essential that they maintain their energy production through either the glycolytic pathway or oxidative phosphorylation. The process of cryopreservation induces cellular damage, leading to a reduction in energy generation and a consequent decline in motility. The assessment of energy production and the efficacy of the freezing process involves the consideration of mitochondrial membrane potential as a significant parameter (Alamo et al. 2020, Wang et al., 2003). Upon examination of the findings from our study, it is apparent that the utilization of insulin in elevated quantities results in an increase in the potential of the mitochondrial membrane (in I-20) compared to the control group. Also, the data related to the insulin group administered at a dosage of 15 IU exhibited a numerical escalation. These findings are consistent with the previously mentioned information. The augmentation in motility has been verified to be attributed to the favorable impact on energy metabolism. The study carried out by Onder et al. (2022), used alpha lipoic acid, a compound involved in energy metabolism, and revealed a beneficial impact on spermatological parameters during the process of cryopreservation. Furthermore, a noteworthy reduction in these parameters was observed in comparison to the control group after a 6-hour incubation period. The study's authors posited that the observed phenomenon could potentially be attributed to heightened levels of reactive oxygen species resulting from elevated energy metabolism. The authors posit that it is crucial to consider the prolonged utilization of raising insulin dosages.

CONCLUSION

In conclusion, the administration of insulin lispro at adequate dosages to ram semen extenders was seen to have favorable outcomes in terms of motility and plasma membrane integrity during cryopreservation. The administration of higher doses of insulin lispro resulted in a statistically significant rise in mitochondrial membrane potential. However, this increase had a detrimental impact on the integrity of the acrosome.

Conflict of Interest: The authors declare that they have no conflict of interest.

Author Contribution: NTO designed study and wrote the manuscript. NTO, TG, SY and YO performed spermatological analysis. NTO performed the statistical analysis.

Ethics Committee Information: All issues associated to the experimental setups and evaluation techniques have been approved by The Scientific Ethical Committee at the Kafkas University in Kars, Türkiye (2021–196).

Financial Support: This study was supported by the Kafkas University Scientific Research Projects Unit, Kars, Türkiye (BAP) (Project number: 2022-TS-55).

REFERENCES

Aitken RJ, Curry BJ, Shokri S, Pujianto DA, Gavriliouk D, Gibb Z, Whiting S, Connaughton HS, Nixon B, Salamonsen LA, Baker MA. Evidence that extrapancreatic insulin production is involved in the mediation of sperm survival. Mol Cell Endocrinol. 2021; 526: 111193.

Alamo A, De Luc C, Mongioì LM, Barbagallo F, Cannarella R, La Vignera S, Calogero AE, Condorelli RA. Mitochondrial membrane potential predicts 4-hour sperm motility. Biomedicines. 2020; 8 (7): 196.

Alcay S, Aktar A, Koca D, Kilic MA, Akkasoglu M, Sagirkaya H. Positive effect of autologous platelet rich plasma on Saanen buck semen cryopreservation in non-breeding season. Cryobiology. 2021; 103: 45-48.

Alçay S, Cakmak S, Cakmak I, Aktar A, Ustuner B, Yilmaz M, Duman M, Ozkan H, Akkaya Y, Sağırkaya H, Nur, Z. Honey bee drone (Apis mellifera) sperm cryopreservation with rainbow trout seminal plasma supplemented extenders. J Hellenic Vet Med Soc. 2022; 73 (1): 3751-3756.

Alcay S, Gokce E, Toker MB, Onder NT, Ustuner B, Uzabacı E, Gul Z, Cavus S. Freeze-dried egg yolk based extenders containing various antioxidants improve post-thawing quality and incubation resilience of goat spermatozoa. Cryobiology. 2016; 72 (3): 269-273.

Aquila S, Giordano F, Guido C, Rago V, Carpino A. Insulin affects sperm capacity in pig through nitric oxide. Asian J Androl. 2013; 15 (6): 835-837.

Bruning JC, Gautam D, Burks DJ, Gillette J, Schubert M, Orban PC, Klein R, Krone W, Müller-Wieland D, Kahn CR. Role of brain insulin receptor in control of body weight and reproduction. Science. 2000; 289 (5487): 2122-2125.

Dias TR, Alves MG, Silva BM, Oliveira PF. Sperm glucose transport and metabolism in diabetic individuals. Mol Cell Endocrinol. 2014; 396 (1-2): 37-45.

Gürler H, Malama E, Heppelmann M, Calisici O, Leiding C, Kastelic JP, Bollwein H. Effects of cryopreservation on sperm viability, synthesis of reactive oxygen species, and DNA damage of bovine sperm. Theriogenology. 2016; 86 (2): 562-571.

Holleman F, Hoekstra JB. Insulin lispro. N Engl J Med, 1997; 337 (3): 176-183.

Karamitsos DT. The story of insulin discovery. Diabetes Res Clin Pract. 2011; 93: 2-8.

Koyunlu A. Spor ve Egzersiz Metabolizmasına Güncel Bakış, In: İnsülin Metabolizması ve Egzersiz İlişkisi, Ed; Pancar Z, 1st Ed., İstanbul, 2023; pp. 73-89.

Lampiao F, Du Plessis SS. Insulin and leptin enhance human sperm motility, acrosome reaction and nitric oxide production. Asian J Androl. 2008; 10 (5): 799-807.

Lunze K, Singh T, Walter M, Brendel MD, Leonhardt S. Blood glucose control algorithms for type 1 diabetic patients: A methodological review. Biomed Signal Process Control. 2013; 8 (2): 107-119.

Noble SL, Johnston E, Walton B. Insulin lispro: a fast-acting insulin analog. American Family Physician, 1998; 57 (2): 279-286.

Onder NT, Alcay S, Nur Z. Effects of alpha-lipoic acid on ram semen cryopreservation and post-thaw life span. Andrologia. 2022; 54 (1): e14249.

Silvestroni L, Modesti A, Sartori C. Insulin-sperm interaction: effects on plasma membrane and binding to acrosome. Arch Androl. 1992; 28 (3): 201-211.

van Tilburg MF, de Oliveira RV, Gadelha CRF, Fagundes B, Dias AJB, Quirino CR, Silva JSF. Effects of insulin on sperm cell quality in ram semen cooled at 5° C. Semina Cien Agrar, 2021; 42 (6): 3803-3812.

Vazquez JM, Martinez EA, Martinez P, Garcia-Artiga C, Roca J. Hypoosmotic swelling of boar spermatozoa compared to other methods for analysing the sperm membrane. Theriogenology. 1997; 47 (4): 913-922.

Wang X, Sharma RK, Gupta A, George V, Thomas Jr AJ, Falcone T, Agarwal A. Alterations in mitochondria membrane potential and oxidative stress in infertile men: a prospective observational study. Fertil Steril. 2003; 80: 844-850.

Weerakoon, W. W. P. N., Sakase, M., Kohama, N., & Kawate, N. Plasma estradiol-17β, cortisol, and insulin concentrations and serum biochemical parameters surrounding puberty in Japanese Black beef bulls with normal and abnormal semen. Theriogenology. 2020; 148: 18-26.

Yildiz S, Öztürkler Y, Ari UC, Lehimcioglu NC, Atakisi E, Kulaksiz R. The effects of l-ergothioneine, n-acetylcystein and cystein on freezing of ram semen. Kafkas Univ Vet Fak Derg. 2015; 21 (1): 81-86.