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RESEARCH ARTICLE

Insulin and Bull Sperm Interactions During Cryopreservation

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ABSTRACT

The aim of the present study was to evaluate the effects of insulin supplemented extender on bull semen at a post-thaw stage. Semen samples were collected four times from a Zavot bull which is endangered species in Türkiye. Semen samples were diluted with Tris based extender supplemented with 10 IU or 15 IU of insulin and control. Motility and plasma membrane integrity were evaluated by a phase-contrast microscope. Acrosome integrity (Fitc-Peanut Agglutinin), mitochondrial membrane potential (Rhodamine 123), nitric oxide level (4,5-Diaminofluorescein-2/diacetate) were evaluated with dual staining propidium iodide using flow cytometry. Motility and plasma membrane functional integrity were better preserved compared to control group ($P<0.05$). Acrosome integrity results were statistically similar between control and 10 IU insulin groups ($P>0.05$), but increased insulin negatively affected acrosome integrity ($p<0.05$). Mitochondrial membrane potential was found to be higher when compared all cells in insulin containing groups compared to control ($P<0.05$). However, results were found similar in all groups with PI negative/mitochondrial membrane potential positive ($P>0.05$). There were not any significant differences among groups in terms of nitric oxide level ($P>0.05$). In conclusion, it was thought that insulin involved in energy metabolism and improved preservation of bull semen.

Keywords: Zavot, Bull semen, Insulin, Cryopreservation

INTRODUCTION

Embryos and germ cells are frozen and stored in order to ensure the continuance of fertility in all living species. The storage of genetic resources for this reason may avert future fertility declines and, perhaps, the extinction of a race or species [1]. Accordingly, the preservation of gamete cells in endangered species, such as the Zavot cow, which is endangered in Türkiye, is of particular relevance [1-3].

In many cases, frozen sperm is used in assisted reproduction. Many investigations are conducted on the composition and effects of semen extenders in an effort to lessen the deleterious effects of freezing and thawing on semen and so boost its reproductive potential [4-6]. In these research, fertility-related characteristics like as motility and acrosome integrity are evaluated [7].

Insulin enhances sperm motility through participating in glucose and lipid metabolism, and it also affects the plasma membrane and acrosome of sperm [9,10]. It has been shown that insulin given to several species' sperm extenders improves sperm motility and reduces the negative effects of freezing phases [9,11,12]. The purpose of the current study is to explore the effects of freezing bull sperm and insulin at post-thaw stage.

MATERIAL AND METHODS

Ethical Approval

Animal Experiments Local Ethics Committee of Kafkas University (Approval number: KAÜ-HADYEK/2021-194) have approved all issues concerning the experimental setups and evaluation techniques.

Animals

A Zavot bull of the 3 years old was maintained at Prof. Dr. Ali Riza Aksoy Education, Research and Application Farm at Faculty of Veterinary Medicine, Kafkas University, Kars, Türkiye.

Chemicals

4,5-Diaminofluorescein-2/diacetate (DAF-2/DA) was

from Genaxxon Bioscience (Biberach, Germany). Other chemicals used in the study were obtained from Merck (Merck, Darmstadt, Germany) and Sigma (Sigma, USA) unless those mentioned.

Experimental Design

The study was constructed with a total of three groups: those with 10 IU and 15 IU of insulin added to the extenders, as well as the control group. 10 IU (I-10) and 15 IU (I-15) insulin lispro (Humalog, Lilly, Italy) added on 3 mL of extenders. Extenders consisted of 223.7 mmol/L Tris, 55.5 mmol/L fructose, 66.6 mmol/L citric acid, 100.4 mmol/L Trehalose, 4.03 mmol/L EDTA (Merck), 4 g/L penicillin G, 3 g/L dihydrostreptomycin, 20% egg yolk (v/v) in distilled water.

Semen collection was performed four times every other day using artificial vagina. After the collection, the ejaculates were transferred to a water bath (37°C). Rapid wave motion and motility was evaluated by a phase-contrast microscope (Nikon Eclipse-E400, Tokyo, Japan) with a warm slide (37°C). Ejaculates with > 70% motility and > $1.5x10^9$ spermatozoon/mL were chosen for cryopreservation.

Each of the groups was diluted to $25x10^6$ sperm/mL with related extender. The groups were then cooled to 5°C within 1 h. Following equilibration, the straws were subjected to cryopreservation by exposure to liquid nitrogen vapor at a distance of 4 cm above the surface of the liquid nitrogen, which maintained a temperature of -120°C, for a duration of 15 min. Subsequently, the straws were immersed in liquid nitrogen for preservation. The cryopreserved semen contained within straws underwent a thawing process at a temperature of 37°C for a duration of 30 sec within a water bath, in order to facilitate subsequent evaluation following the thawing process [7,13].

Semen Analysis

The motility of sperm was evaluated subjectively using a 400x phase-contrast microscope with a 37°C warm slide [26]. The functional integrity of the sperm membrane was assessed through the use of Hypo osmotic swelling test (HOST) [14], which involved the incubation of 10 µL of semen with 100 µL of 100 mOsm hypoosmotic solution (9 g fructose + 4.9 g sodium citrate per liter of distilled water) at 37°C for 60 min. The evaluation was based on the observation of curled and swollen tails. Following incubation, a volume of 20 µL of the aforementioned mixture was applied onto a heated slide and subsequently covered with a slip. Two hundred sperm cells were examined using a phase-contrast microscope at a magnification of 1000x. Spermatozoa exhibiting morphological anomalies such as tail swelling or coiling were documented.

Flow Cytometric Analysis

Analysis was performed using Attune NxT Acoustic Focusing Cytometer (Invitrogen, USA). Fluorescence was measured at 480 nm excitation (10 nm excitation bandwidth) and 530/30 nm filter (BL-1) and 695/40 nm filter (BL-3) emission connected to Attune NxT software v2.7 (Thermo Fisher). After gating the cell population using forward and side scatter light signals, the average fluorescence intensity of the sperm cells under analysis was measured. There were 10.000 sperm cells in the per assay.

The fluorescein isothiocyanate–conjugated peanut agglutinin (PNA)/propidium iodide (PI) dual-staining technique was used to evaluate acrosome integrity. Rhodamine 123/PI was used to evaluate mitochondrial membrane potential. DAF-2/DA / PI was used to evaluate nitric oxide level in live cells. All flow cytometric analysis performed as previously described by Gürler et al.[15].

Statistical Analysis

IBM SPSS version 28 was used to do statistical analysis. The Shapiro Wilk test was used to assess for normality. The data were shown as mean ± standard error. The statistical significance of the differences between subdivided groups was determined using one-way ANOVA followed by Tukey. The Kruskal-Wallis test was used to examine data having a non-normal distribution. P values less than 0.05 were deemed statistically significant.

Results

Motility, plasma membrane functional integrity, acrosome integrity, mitochondrial membrane potential, and nitric oxide concentration are detailed in *Table 1*.

The post-thaw motility was found to be 47.08, 55.00 and 52.00 in control, I-10 and I-15 groups, respectively. Motility was better preserved in groups containing insulin ($P<0.05$). It was observed that the semen dilutions with extender containing insulin protected the plasma membrane integrity better than the control group ($P<0.05$). In living cells, the overall acrosome integrity and the acrosome integrity ratio were equivalent in the control and I-10 groups ($P>0.05$), but considerably lower in the I-15 group ($P<0.05$). By analyzing all cells, it was shown that mitochondrial membrane potential was higher in insulin-containing groups ($P<0.05$). All groups exhibited a similar ratio of mitochondrial membrane potential in living cells ($P>0.05$). Nitric oxide levels in living cells did not differ significantly among the groups ($P>0.05$).

Discussion

Motility of spermatozoa is one of the most critical measures in evaluating male reproductive ability [16].

Table 1. *Effects of insulin on spermatological parameters*

Groups	Measurements						
	Motility (%)	**HOST (%)**	**A (%)**	**A-P (%)**	**M (%)**	**M-P (%)**	**NO (%)**
Control	47.08±1.30[a]	73.25±1.30[a]	75.12±1.27[a]	63.06±1.89[a]	71.84±2.89[a]	37.84±1.64	41.72±1.68
I-10	55.00±1.51[b]	79.00±0.69[b]	74.35±1.47[a]	60.09±1.43[a]	81.82±2.29[b]	38.21±2.25	37.43±1.74
I-15	52.00±1.14[b]	77.67±0.87[b]	70.33±1.30[b]	53.08±1.73[b]	85.27±1.29[b]	38.44±1.79	38.07±1.64

[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,* ***NO:*** *Nitric Oxide Positive with Intact Plasma Membrane*

Diabetes mellitus type-1 has been observed to negatively impact sperm motility in males [11]. Furthermore, glucose-free media was found to suppress the acrosome response of human spermatozoa [8]. Spermatozoa generate metabolic activity depending on an insulin-dependent signaling mechanism [22]. As a matter of fact, with the discovery of insulin receptors in spermatozoa in recent years, the complex mechanism of how spermatozoa can metabolize sugars, amino acids and fatty acids has been shed light on. In the current investigation, motility was shown to increase considerably in the groups containing insulin. It is believed that the reduction in unfavorable effects of the freeze-thaw process is related to insulin's ability to boost the use of energy sources. Sugars added to sperm extenders create osmotic pressure and inhibit the development of ice crystals during freezing. And, the sperm metabolism is slowed by the chilling procedure before the freezing process [17,18]. The interesting aspect of our study is that spermatozoa's potential absorption of sugar sources may increase metabolic activities and the effectiveness of cooling and freezing processes.

There is a correlation between sperm membrane functional integrity and motility, according to studies [7,13,14]. Fertilization, the most fundamental success criterion of sperm freezing, requires that the functional integrity of the sperm membrane be maintained throughout the freezing and thawing processes. The hypo-osmotic swelling test is used to evaluate the plasma membrane integrity; it examines the swelling and curling of spermatozoa with an active biochemical structure in an environment containing water. Researchers prefer this test because it is reliable and affordable [7,16,25]. The integrity of the plasma membrane was better preserved in the insulin-containing groups, as evidenced by our study, which is consistent with the motility results.

In order for sperm freezing to be successful, it is crucial to maintain the integrity of the acrosome, which plays a crucial role in the fertilization [16]. In our investigation, 10 IU of insulin resulted in acrosome integrity comparable to that of the control group, but a high dose had a deleterious effect on acrosome integrity. According to research on human spermatozoa, spermatozoa have insulin receptors in the acrosome area and trigger the acrosome reaction [10,11]. In light of this knowledge, it is believed that raising the insulin dosage in the trial at hand increases the acrosome reaction.

A positive correlation exists between elevated mitochondrial membrane potential and increased motility [19]. However, mitochondrial membrane potential decreases in time after sperm retrieval, especially in humans, and it has been stated that mitochondrial membrane potential can be used to predict motility in humans [23]. In our research, insulin groups had a high percentage of total mitochondrial membrane potential. This is consistent with the increased motility seen in the insulin-treated groups and the function of insulin in glucose and lipid metabolism. The mitochondrial membrane potential data of live spermatozoa, however, did not reveal any significant differences.

It is also a known fact that nitric oxide plays a critical role in male fertility. Nitric oxide can regulate spermatogenesis, sperm maturation and motility, and the apoptosis process in abnormal germ cells, as well as decrease sperm motility and cause sperm toxicity and infertility [24]. According to the research conducted by Lampiao and Du Plessis [11], insulin promotes an increase in the concentration of nitric oxide in human sperm. Moreover, it has been reported that L-arginine stimulates the nitric oxide production [20]. Hoshiyama et al.[21] reported in their work on Human Glomerular Endothelial Cells that an increased level of glucose in the medium inhibits nitric oxide production, and the addition of L-arginine to the medium weakens this impact of glucose. With this information, when the presence of L-arginine in the diluent content used by Lampiao and Du Plessis is evaluated, it is thought that the increase in the amount of nitric oxide in their study might be caused by L-arginine. Furthermore, insulin added to human sperm extender has an antioxidant effect and decreases the number of reactive oxygen species, according to another research [12]. In the presented

investigation, it was shown that the addition of insulin had no influence on the level of nitric oxide. On the other hand, a value below that of the control group was discovered. The plausible causes for this situation, as posited by the authors, include a higher concentration of carbohydrates in our investigation compared to the study conducted by Lampiao and Du Plessis [11], the L-arginine content in Ham's F10 medium as observed in the study by Lampiao and Du Plessis, differences in the content of the diluent, and variations in species.

In conclusion, it was found that insulin had positive effects on motility, plasma membrane integrity and mitochondrial membrane potential, but increased dose had a negative effect on acrosome. Overall results have been shown that 10 IU insulin facilitates the effective freezing of bull sperm and may have positive impacts on potential fertility.

Availability of Data and Materials

The data used to support the findings of this study are available from the corresponding author upon request.

Ethical Approval

Animal Experiments Local Ethics Committee of Kafkas University (Approval number: KAÜ-HADYEK/2021-194) have approved all issues concerning the experimental setups and evaluation techniques.

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This study was not financially supported.

Conflict of Interest

The authors declare that they have no conflict of interest.

Author Contribution

NTO designed study and wrote the manuscript. NTO analyzed post-thaw motility and flow cytometry results. TG performed HOST. CK and MCD provided the bull. SY and YÖ performed semen analysis at semen collection. NTO, MCK and OS performed cryopreservation process. NTO performed the statistical analysis.

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