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Research Article

Lyophilized Extender Supplemented with Rainbow Trout *(Oncorhynchus mykiss)* Seminal Plasma Improves Cryopreservation of Ram Sperm

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Abstract: This study aimed to investigate the effect of the Rainbow trout seminal plasma (RTSP) supplemented (control, 10% or 15%) lyophilized extender on freezability of ram semen. Collected semen was pooled and split into two aliquots, and each of the ejaculates was diluted with fresh or lyophilized extenders with RTSP (0%, FC and LC; 10% F10 and L10or 15% F15 and L15) using two-step dilution method. Semen was frozen using the programmable freezing machine. Semen samples were examined for sperm motility, defective acrosomes, plasma membrane integrity and DNA fragmentation at native and post-thaw stage. The highest percentages of post-thaw motility and plasma integrity were observed in the F10, F15, and L15 ($P<0.05$). There was no significant difference in the rate of post-thaw defective acrosomes when the other extender groups were compared with the control. The highest percentage of post-thaw DNA fragmentation values were observed in the FC group, while the lowest DNA fragmentation was obtained in the F15 but only significant different from FC and LC groups. In conclusion, the findings of this study show that the lyophilized extender with 15% RTSP added can be used successfully for freezing ram semen.

Keywords: *Cryopreservation, Lyophilized extender, Rainbow trout seminal plasma, Ram semen*

Gökkuşağı Alabalığı *(Oncorhynchus mykiss)* Seminal Plazması İlave Edilmiş Liyofilize Sulandırıcı, Koç Spermasının Dondurulmasını İyileştirir

Öz: Bu çalışma Gökkuşağı alabalığı seminal plazması (ASP) ilave edilmiş (kontrol, %10 ve %15) liyofilize sulandırıcının koç spermasının dondurulabilirliği üzerindeki etkisini belirlemeyi amaçlamıştır. Alınan sperma birleştirilerek, iki eşit hacime bölündü ve ejakülatların her biri, iki aşamalı sulandırma yöntemi kullanılarak ASP (%0, FC ve LC; %10 F10 ve L10 veya %15 F15 ve L15) ilave edilen taze ve liyofilize sulandırıcılarla sulandırıldı. Sperma programlanabilir dondurma makinesi kullanılarak donduruldu. Sperma numuneleri, taze ve eritme sonrası aşamada motilite, akrozom hasarı, plazma membran bütünlüğü ve DNA fragmentasyonu bakımından incelendi. Eritme sonrası en yüksek motilite ve plazma membrane bütünlüğü F10, F15 ve L15 gruplarında gözlendi ($P<0.05$). Eritme sonrası motilite değerlerine paralel olarak %10 ASP içeren liyofilize sulandırıcının plazma membran bütünlüğü, kontrol gruplarından daha yüksek bulundu. Sulandırıcı grupları kontrol grupları ile karşılaştırıldığında, eritme sonrası akrozom bozukluğu bakımından istatistiksel bir fark tespit edilmedi. Eritme sonrası DNA fragmentasyonu en yüksek FC grubunda gözlenirken, en düşük F15 grubunda elde edildi fakat yalnızca FC ve LC gruplarından istatistiksel olarak farklıydı ($P<0.05$). Sonuç olarak, bu araştırmanın bulguları %15 ASP ilave edilen liyofilize sulandırıcının koç spermasının dondurulmasında başarıyla kullanılabileceğini göstermektedir.

Anahtar sözcükler: *Dondurarak saklama, Gökkuşağı alabalığı seminal plazması, Koç sperması, Liyofilize sulandırıcı*

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Introduction

Although researchers have tried to freeze ram semen with different protocols and diluents for years, the fertility rates obtained with frozen semen are not as high as those acquired with fresh semen [1,2]. The reason for the reduction in fertilization rate of ram sperm is the decrease in motility, increase in acrosomal damage, and adversely affecting chromatin integrity in the post-thaw stage [3-6].

Increased levels of reactive oxygen species (ROS) due to cold shock origin changes in cellular functions and increase the susceptibility of semen to oxidative damage [7]. Thus, the migration ability of frozen-thawed ram semen in the female genital tract decreases, and the fertility rates after cervical insemination are not at the desired level [8,9].

Studies to ameliorate the maintenance of ram semen are concentrated on modifying extenders by adding various components to preserve sperm motility, membrane integrity, and fertilization capacity [3,10,11].

Previous studies of minimizing the effect of cryo-damage on spermatozoa have dealt with the addition of various complements such as seminal plasma of the same or different species in the freezing extenders or thawing solutions [12-14]. Seminal plasma comprises notable biochemical constituents that are responsible for regulating sperm function [15]. Supplementation of rainbow trout seminal plasma (RTSP) to egg-yolk-based extenders used cryopreservation of ram and goat semen successfully [13,16]. Unlike mammals, since Rainbow trout do not have accessory glands, RTSP does not originate from the accessory reproductive gland but the blood plasma and cuboidal cells in the semen duct [17]. Monosaccharides and triglycerides in RTSP form the energy source of spermatozoa and maintain the viability of these cells [18]. RTSP proteins, on the other hand, are essential in terms of helping the motility ability and maintaining the viability of the spermatozoon [19]. Shaliutina-Kolešová et al.[20] reported that about 25% of the proteins identified in the RTSP are multifunctional proteins (apolipoprotein, transferrin, serum albumin), immune response-related proteins (complements), antioxidant proteins (SOD, glutathione-S-transferase, thioredoxin, etc.), cold-shock proteins, enzymes (glyceraldehyde-3-phosphate dehydrogenase, lactate dehydrogenase, creatine kinase) and 14-3-3 family members. At the same time, some specific proteins, such as type-4 ice-formatting protein, known as LS-12 anti-freeze protein, isolated in RTSP prevent the freezing of fish semen in low-temperature waters [17,21]. Due to all these properties of rainbow trout seminal plasma, it has been commented that adding it to the extender positively affects the freezability of ram semen [13].

Lyophilization is mainly used to remove the water from sensitive biological origin products without any damage. Thus these sensitive products can be preserved, permanently stored, and be reconstituted smoothly by water addition [22]. Chemicals, biotechnological products, bacteria, cells and tissues, diagnostic drugs, antibiotics, and vaccines are examples of products provided by lyophilization technology. Therefore, the extender supplemented with RTSP, which has proven successful in freezing ram semen, can be lyophilized and stored later in semen cryopreservation.

This study aimed to investigate the effect of the RTSP supplemented (control, 10% or 15%) lyophilized and fresh extender on post-thaw ram spermatological parameters (motility, plasma membrane, acrosome, and DNA integrities).

Material and Methods

Ethical Approval

The Scientific Ethical Committee (Bursa Uludag University, Bursa, Turkey, Approval No: 2020-05/03) accepted all protocols regarding the experimental design and evaluation procedures planned in the research.

Semen Extender Preparation

Rainbow trout milt was collected by abdominal massage using the method of Glogowski et al.[23] Subsequently, RTSP obtained from milt using the method of Ustuner et al.[13] was aliquoted and stored for up to 1 month at -20°C in the eppendorf tubes until added to the extender.

The extenders to be used in the experiment were designed to be fresh (control) and lyophilized. Both fresh and lyophilized extenders were prepared by a two-stage dilution method. Tris-based extenders were prepared by the addition of 20% egg yolk (EY), and Extender A did not contain glycerol. The freezing extender (Extender B) was composed of Extender A with added EDTA (0.15 g/100 mL), trehalose (3.8 g/100 mL), and glycerol 6% (v/v) [24]. In order to achieve successful lyophilization of the lyophilized group, glycerol was added to the lyophilized groups (extender B) after the lyophilization process.

RTSP's miscellaneous concentrations (0%, 10%, or 15%) were added to the groups of extender A and B with respect to the experimental design. Extenders (fresh (F) or lyophilized (L)) were designated as follows: FC, (no RTSP); F10, 10% RTSP; F15, 15% RTSP and LC (no RTSP); L10, 10% RTSP and L15, 15% RTSP.

Lyophilization of the extenders was performed according to the procedure previously described by Alcay et al.[25] After the preparation process, each extender group was lyophilized using Freeze-Dryer (Labconco, Kansas City, U.S.) Subsequently, prepared extenders were stored at 4°C until use.

Semen Collection, Evaluation, and Dilution

Three to 5 aged five rams from Bursa Uludag University,

Faculty of Veterinary Medicine in Bursa were selected for semen collection using electrically stimulated ejaculation method twice a week during the non-breeding season (Ruakura Ram Probe Plastic Products, Hamilton, New Zealand) [3]. The semen collection process was repeated five times.

Collected semen was immediately evaluated and ejaculates with a thick consistency, a rapid wave motion (3-5 on a 0-5 scale), and >70% initial motility were pooled [13].

Pooled ejaculates were split into two aliquots, and each of the ejaculates was diluted to a ratio of 1:2 (semen/extender) with fresh or lyophilized extenders A (0%, 10% or 15% RTSP). Subsequently, cooled to 5°C within 1 h. The cooled semen groups were then diluted to a 1:1 (semen/extender) ratio with fresh and lyophilized extenders B (5°C), respectively. Equilibration was performed for diluted semen samples at 2 h at 5°C.

Semen Freezing and Thawing

Equilibrated semen was placed into 0.25 mL straws and frozen at 3°C/min from +5 to -8°C and at 15°C/min from -8 to -120°C in liquid nitrogen vapor using the Nicool Plus PC freezing machine (Air Liquide, Marne-la-Vallée Cedex 3, France) (0.25 mL straw contained $125x10^6$ spermatozoa). The frozen straws were immersed in liquid nitrogen (-196°C), where they were stored for at least one month. To perform post-thaw semen evaluations, leastwise three straws from each group were thawed at 37°C for 30 sec in the water bath.

Semen Evaluation

All semen parameters were assessed at the following two-time points: native and post-thaw stages.

Semen freezing and evaluation of semen parameters were performed by the same person throughout the study. Sperm motility was assessed by using a heated mechanical stage attached phase-contrast microscope (Olympus BX51) subjectively [26].

Fluorescein Lectin Staining Assay (FITC Conjugated *Pisum sativum* Agglutinin (FITC-PSA)

Assessment of acrosome integrity was performed using the method of Ustuner et al.[14] According to the procedure, stained slides were assessed under a fluorescent microscope, and at least 200 spermatozoa were counted for evaluation.

The Hypoosmotic Swelling Test (HOST)

Evaluation of the functional integrity of the sperm membrane was performed by incubating 10 µL of semen with 100 µL of a 100 mOsM hypoosmotic solution. After incubation at 37°C for 60 min., 200 spermatozoa were assessed under phase-contrast microscopy, and coiled or swollen tails were noted [27].

TUNEL (Terminal-Deoxynucleotidyl-Transferase-Mediated-dUTP Nick-End Labelling) Assay

For the assessment of DNA integrity, the manufacturer's slight-modified TUNEL technique was performed [3]. After the labeling process, slides were analyzed via fluorescence microscopy, and leastwise 100 spermatozoa were assessed to determine the TUNEL positive sperm percentage.

Statistical Analysis

All data obtained from the study were analyzed using SPSS. (SPSS 20.0 for Windows; SPSS, Chicago, IL, USA). Shapiro Wilk test was used as a normality test. According to normality and homogeneity tests, the data which present normal distribution was analyzed using One Way Anova Test, and Tukey was used as a Posthoc test. The semen parameters presented nonparametric distribution was analyzed using the Kruskal Wallis test followed by the Mann Whitney U test.

Results

Motility, plasma membrane integrity, defected acrosome and DNA fragmentation rates of pooled fresh semen samples were 77±2.7%, 85.2±3.2%, 14.4±5.2%, and 2.2±0.8%, respectively.

The effects of the fresh and lyophilized diluents containing different concentrations of RTSP at post-thaw on ram sperm motility, plasma membrane, acrosome, and DNA integrity are presented in *Table 1*.

The freeze-thaw process diminished sperm motility

Table 1. *Mean motility, plasma membrane integrity, defected acrosome and DNA fragmentation rates in post-thawed ram semen. (mean±SEM)*

Groups	n	Motility (%)	Plasma Membrane İntegrity (%)	Defected Acrosome (%)	DNA Fragmentation (%)
FC	15	44.29±1.56^{a}	70.40±2.38^{a}	39.8±3.02	13.0±0.71^{a}
F10%	15	52.67±1.28^{b}	75.6±1.36^{b}	36.40±6.08	9.6±0.51^{bc}
F15%	15	54.23±1.59^{b}	76.00±4.32^{b}	38.00±4.38	8.80±0.38^{c}
LC	15	41.07±1.83^{a}	63.20±6.17^{a}	36.60±3.03	10.60±0.75^{b}
L10%	15	46.15±1.29^{ac}	68.00±3.90^{a}	34.00±1.92	9.00±0.45^{bc}
L15%	15	51.67±1.42^{bc}	76.80±3.22^{b}	38.60±3.56	10.00±0.90^{bc}

^{a,b,c} *Different superscripts within the same column are significantly different (P<0.05)*

progressively (P<0.05). The highest percentages of post-thaw motility were observed in the F10, F15, and L15, and these rates were significantly higher than observed in the control groups (P<0.05). It was observed that the motility of the L10 group was numerically higher than the FC and LC groups, although not statistically (P>0.05).

The plasma membrane functional integrity was affected by the freeze-thaw process negatively (P<0.05). Higher percentages of post-thaw plasma integrity were observed in the F10, F15, and L15 groups than in the other groups (P<0.05). In parallel with the post-thawing motility values, the plasma membrane integrity of the lyophilized diluent containing 10% RTSP was found to be slightly higher than the LC group.

Acrosome integrity deteriorated during the freeze-thaw process but there was no significant difference in the rate of post-thaw defective acrosomes when the other extender groups were compared with the control (P>0.05).

Sperm DNA integrity was negatively affected by freeze-thaw process. The highest percentage of post-thaw DNA fragmentation values were observed in the FC group (P<0.05). Although the lowest DNA fragmentation was obtained in the F15 group, it was only statistically different from the control groups of fresh and lyophilized extenders (P<0.05).

Discussion

Although cattle and pigs are the most commonly used livestock of artificial insemination biotechnology world-wide; In sheep farming, since acceptable pregnancy rates cannot be obtained with frozen semen, artificial insemination practices cannot be utilized to the same extent. The most important reason is that ram semen is much more sensitive to freezing processes than other farm animals. In many studies conducted in recent years, it has been revealed that seminal plasma proteins have various effects on the freezing of semen [12-14,28]. The current study, therefore, set out to assess the effect of the lyophilized extender with added rainbow trout seminal plasma on ram sperm freezability.

The freeze-thaw process of semen causes irreversible damage to motility, acrosome, plasma membrane, and DNA integrity [3,24,29]. Comparing fresh pooled semen to post-thaw results of the current study, predictably acrosome, plasma membrane, and DNA integrities were affected by the cryopreservation process negatively (P<0.05).

Spermatozoa must have motility to progress through the female genital canal to reach the fertilization area and penetrate the zona pellucida (ZP) with the cumulus cells on the oocyte surface [30]. Sperm motility is affected by many factors such as the content and osmotic pressure of the extender used in the cryopreservation process, cooling rate, cold shock, freezing rate, presence of ROS, thawing temperature, and time. In the current study, post-thaw motility of the lyophilized-extender supplemented with 15% RTSP and freshly prepared extender groups with 10% and 15% RTSP (P<0.05) were statistically higher than the control groups. The positive effect of RTSP on the post-thaw success of ram semen of lyophilized extender was in good agreement with Ustuner et al.[13] and Alcay et al.[16] This positive effect is probably due to the presence of cathepsin D and M, calpain, cytosolic nonspecific dipeptidase, proteasome, and antifreeze protein (type-4 ice-structuring protein [LS-12]) in RTSP. Among the mentioned proteins, especially LS-12 is known to prevent fish semen from being damaged in cold water conditions, and notably it is not found in mammalian semen [17,21]. It is known that other proteins mentioned above are important in stimulating motility and eliminating metabolic wastes of damaged spermatozoa [31]. However, it was observed that the post-thaw motility of the 10% RTSP supplemented lyophilized group was similar to the control groups. This suggests the possibility that RTSP may be inactivated by lyophilization when used at low rates.

The ability of spermatozoa to exchange substances with the external environment is necessary for its physiology. This phenomenon is only possible if the plasma membrane functional integrity is preserved [32]. During cryopreservation, cold shock, osmotic pressure change, ice crystals, and ROS formation cause lipid composition changes in the cell membrane, resulting in morphological damage and loss of function in the plasma membrane [33]. Nur et al.[3] reported that there is a correlation between sperm motility and functional plasma membrane integrity. In our study, it was observed that the plasma membrane integrity of this study groups was parallel and associated with motility. Furthermore, the post-thaw functional sperm plasma membrane integrity in lyophilized extender supplemented with added RTSP was found higher than the findings of Alcay et al.[25].

As mentioned in the literature reviews, acrosome reactions or premature capacitation are induced by cryo-preservation [34]. The acrosome defect of 35-65% caused by the freeze-thaw process reported in several previous studies is consistent with the data obtained in the study [13,29,35,36]. There was no significant difference in the rate of post-thaw defective acrosomes when the other extender groups were compared with the control.

The protection of the physiological and morphological integrity of the paternal DNA, with its compact structure, is of great importance for the formation and development of the embryo [37]. In spermatozoa, minimal DNA damage is reversible and does not pose a significant threat to

fertilization, while severe DNA damage causes sterility [38]. This negative effect of DNA damage on fertility is essential in interrupting the viability and development of the embryo rather than reducing the fertilization ability of the sperm [39]. Ram spermatozoa are more susceptible to DNA damage during the thawing process after cryopreservation compared to other species [3,26]. Therefore, the evaluation of DNA integrity is esteemed to determine post-thaw semen quality. The DNA fragmentation rates obtained in the current study were consistent with previously reported results by Alcay et al.[25] The highest percentage of post-thaw DNA damage values were observed in the FC group ($P<0.05$). However, the DNA fragmentation rate of the LC group was similar to all groups except for FC and F15 extender groups ($P<0.05$). Generally, according to the current study data, we can infer that the lyophilized group conserved DNA integrity near the freshly prepared group against damage due to cryopreservation ($P>0.05$).

Sheep artificial insemination on the field commonly is depend on the successfully cryopreserved ram semen. It is an advantage that the lyophilization of this extender is more practical than the extender that is prepared fresh daily.

As a result of the current study, it was seen that there was no difference between the freshly prepared extender and the lyophilized extender with 15% RTSP added. Hereby, frozen semen will have the opportunity to be used more widely in the field.

Competing Interests

There is no conflict to interest.

Authors' Contributions

BU experimental design, prepared extender, collected and frozen semen, evaluated native and post thawed spermatological parameters, wrote the manuscript;, SA lyophilization of extender and contrubution to experimental design; EG staining of spermatological smears with fluorescent dyes, statistical evaluation of the findings; MMY semen collection with electro ejaculator and semen frozen and contrubution to preparation of semen extender; AA semen collection with electro ejaculator and semen frozen; OH evaluation of the manuscript in terms of English grammar and statistical evaluation of the findings; MD collected milt from Rainbow trout; NTO semen collection with electro ejaculator; EA the manuscript in terms of English grammar and statistical evaluation of the findings; ZN edited the manuscript scientifically.

Availability of Data and Materials

The authors declare that the data supporting the study findings were obtained from the correspond-ing author (B. Üstüner).

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