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Freezing of Honamli Goat Buck Sperm with Commercial and Laboratory Extenders and Evaluation of In Vitro Spermatological Parameters

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Abstract: This study aimed to evaluate the effectiveness of commercial versus laboratory-made extenders for cryopreserving Honamli buck semen. Semen was collected from three 2-3-year-old bucks and pooled. The pooled semen was divided into two portions: one with seminal plasma removed and diluted with Tris egg yolk-based extender (TEYBE+), and the other with seminal plasma retained, extended with commercial Bioxcell (B), Tris egg yolk-based extender (TEYBE-), and Tris lecithin-based extender (TLEBE). The extended semen was stabilized at +4 °C for 2 hours, cryopreserved by exposure to liquid nitrogen steam at -120 °C for 12 minutes, and frozen. After thawing, motility, viability, plasma membrane and acrosome integrity (PMAI), high mitochondrial membrane potential (HMMP), and mitochondrial reactive oxygen species (MROS) levels were evaluated.
Group B exhibited the lowest post-thaw motility, viability, PMAI, and HMMP values ($p < 0.001$). The highest viability rate was recorded in the TEYBE+ group ($p < 0.05$). TEYBE- showed superior motility, PMAI, HMMP, and viability compared to B, whereas TLEBE did not outperform TEYBE+. The lowest MROS levels were observed in the TEYBE+ group ($p < 0.05$, compared to B). In conclusion, the findings indicated that the separation of the seminal plasma used in the freezing of buck semen had a beneficial impact on the freezing process. Conversely, the B extender, which is commercially employed for freezing bull semen, was ineffective in cryopreservation of the buck semen when compared to extenders containing lecithin and egg yolk.
Keywords: *Bioxcell, Cryopreservation, Flow cytometry, Honamli.*

Honamlı Teke Spermasının Ticari ve Laboratuvarda Hazırlanan Sulandırıcılar ile Dondurulması ve İn Vitro Spermatalojik Parametrelerinin Değerlendirilmesi

Özet: Bu çalışmanın amacı Honamlı Teke spermasının kriyoprezervasyonunda ticari ve laboratuvar sulandırıcılarını etkinliğini değerlendirmektir. Çalışmada 2-3 yaşlarında üç Honamlı tekesinden spermalar toplandı ve birleştirildi. Birleştirilen sperma iki eşit parçaya bölündü; Bir kısım seminal plazma uzaklaştırıldı ve tris yumurta sarısı bazlı sulandırıcı (TYSBS+) ile sulandırıldı; diğer kısım seminal plazması uzaklaştırılmadı ve Bioxcell (B), tris yumurta sarısı bazlı sulandırıcı (TYSBS-) ve tris lesitin bazlı sulandırıcı (TLBS) ticari sulandırıcı ile sulandırıldı. Sulandırılan sperma örnekleri +4 derecede 2 saat boyunca ekilibrasyona ve sıvı nitrojen buharında (-120°C'de 12 dakika) kriyoprezervasyona tabi tutularak donduruldu. Çözdürme işleminden sonra spermaların motilite, canlılık, plazma membranı ve akrozom bütünlüğü (PMAI), yüksek mitokondriyal membran potansiyeli (HMMP) ve mitokondriyal reaktif oksijen türleri (MROS) seviyeleri değerlendirildi.
B grubu, çözdürme sonrası en düşük motilite, canlılık, PMAI ve HMMP değerlerini göstermiştir ($p < 0.001$). En yüksek canlılık oranı TYSBS+ grubunda kaydedilmiştir ($p<0.05$). TYSBS-, B'ye kıyasla daha yüksek motilite, PMAI, HMMP ve canlılık gösterirken, TLBS, TYSBS+'den daha iyi performans sergileyememiştir. En düşük MROS seviyeleri TYSBS+'de gözlenmiştir ($p < 0,05$, B ile karşılaştırıldığında). Sonuç olarak, teke spermasının dondurulması sürecinde seminal plazmanın uzaklaştırılmasının dondurma başarısı üzerinde olumlu bir etkisi olduğu belirlenmiştir. Buna karşılık, boğa spermasının dondurulmasında yaygın olarak kullanılan ticari B sulandırıcısının, teke spermasının kriyoprezervasyonu açısından, yumurta sarısı ve lesitin içeren sulandırıcılara kıyasla etkisiz olduğu tespit edilmiştir.
Anahtar Kelimeler: *Bioxcell, Kriyoprezervasyon, Akış sitometrisi, Honamlı.*

Introduction

The cryopreservation of buck and ram semen is a crucial component of genetic resource management. The dilution process in buck and ram semen significantly influences the efficiency of artificial insemination (AI) and the overall reproductive efficiency. Moreover, the choice of semen diluent is a key factor impacting fertility rates, yet the development of optimal protocols continues to pose a considerable challenge (Ferreira et al., 2014).

Semen dilution is critical for preserving sperm viability and enhancing fertility outcomes. Research has highlighted the significant impact of various diluents and dilution protocols on sperm quality and reproductive success. Notably, studies have demonstrated that yolk-citrate diluents achieve higher pregnancy rates compared to fresh semen, underscoring the importance of optimizing sperm concentration and diluent composition (Ferreira et al., 2014; Madrigali et al., 2021; Mohamed and Moustafa, 2017).

In bucks, the combination of egg yolk and seminal plasma may negatively affect sperm viability, unlike what has been observed in other species (Gangwar et al., 2016). A protein identified in seminal plasma (SBUIII, the glycoprotein BUSgp60) and an enzyme released by the bulbourethral glands (phospholipase-A, also referred to as the egg yolk coagulating enzyme) have been implicated in spermatozoa toxicity in semen diluents derived from milk and egg yolk (Ferreira et al., 2014; Purdy, 2006). When using semen diluents containing egg yolk or milk for goat semen storage, seminal fluid should be removed by centrifugation to minimize the detrimental effects of bulbourethral gland secretions (Purdy, 2006).

These findings underscore the importance of selecting appropriate diluents to enhance sperm survival and fertilization potential during artificial insemination, and the choice of diluent is critical for maintaining sperm quality during the freezing and thawing processes. To maximize survival rates and fertility, protocols for cryopreservation of goat semen must consider species-specific factors, including diluent composition, pH, osmolality, cryoprotectants, and freeze-thaw techniques (Gangwar et al., 2016).

Egg yolk is frequently incorporated into extenders for preserving ram semen through cryopreservation (Alçay et al., 2015; Anand et al., 2014; de Paz et al., 2010; Gholami et al., 2012; Salamon and Maxwell, 2000; Watson, 2000). Although egg yolk is commonly employed, its application is not without challenges, including the risk of pathogen transmission and variability between batches. Additionally, concerns about biosecurity and its impact on sperm analysis have prompted the pursuit of alternative solutions (Aires et al., 2003; Layek et al., 2016).

Soybean lecithin has emerged as an alternative to animal-origin extender as a plant-origin extender. Soybean lecithin has been recognized as an effective substitute for egg yolk in semen extenders used for the cryopreservation of ram and bull sperm. Researchs have demonstrated that extenders formulated with soybean lecithin can achieve equal or better outcomes than those containing egg yolk, particularly regarding post-thaw sperm quality parameters including motility, viability, and fertility (Forouzanfar et al., 2010; Khatun et al., 2021; Masoudi et al., 2016). Additionally, some studies indicates that the effectiveness of Bioxcell (B) in preserving bull spermatozoa is influenced by its composition, particularly the presence of soy lecithin (Akhter et al., 2010; Kaka et al., 2017). Fernández-Novo et al. (2021) demonstrated that B outperformed other extenders in maintaining the viability of spermatozoa at lower temperatures, specifically 5 °C, which is critical for preserving sperm quality during preservation.

This study aimed to assess the effectiveness of B, a commercial semen extender for bulls, in comparison to Tris-lecithin and Tris-egg yolk-based (with and without centrifugation of seminal plasma) extenders in Honamli buck semen.

Material and Method

Animals and Experimental Design

This research was conducted on three Honamli bucks aged 24–36 months. All bucks were kept under identical care and feeding conditions. Semen samples were obtained biweekly throughout the mating season in Burdur, Turkey, using an electroejaculator. The experiment was repeated five times. The study protocol was approved by Local Ethics Committee Animal Experiments of the Burdur Mehmet Akif Ersoy University (approval number 07.12.2020/201-13.11.2024-1388).

Semen Collection and Processing

Macroscopic and microscopic examinations were performed on the semen collected from each buck. Only fresh semen with normozoospermic conditions (concentration ≥ 2.0x 10^9/ml, mass activity ≥ +++3, volume ≥ 1.0 ml and motility ≥ 70% was used. The pooled semen was split into two parts. One portion was left uncentrifuged for seminal plasma separation and was extended with a Bioxcell (2A23440, BioShop, Canada) extender, along with an egg yolk- and soybean lecithin-based (Sigma, P5638) solution. The second semen portion underwent centrifugation to separate the seminal plasma and was then extended with an egg yolk-based solution. The same Tris buffer [299 mM Trizma (Sigma, T1503), 90 mM citric acid (Sigma, C0759), 20 mM glucose (Sigma, G7528) and distiled water] was used in extenders containing egg yolk and lecithin. The final group configurations are outlined in Table 1.

Following dilution, samples were loaded into 0.25 ml straws to achieve a concentration of around 200×10^6 spermatozoa/ml and then equilibrated at +4 °C for two hours. The straws were then placed in liquid nitrogen vapor (approximately 10 cm above the liquid nitrogen, ~ -120 °C) for 12 minutes, followed by storage in a nitrogen tank at -196 °C. The thawing process for spermatological evaluation involved placing semen straws from each experimental group in a 37 °C water bath for 30 seconds, at least two months post-freezing.

Table 1. Compositions of the groups.

Groups	Compositions
Bioxcell (B)	Bioxcell + 6% Glycerol (GLY) (Non-centrifuged semen)
TLEBE-	Tris-based extender + 1% Lecithin (LC) + 6% GLY (Non-centrifuged semen)
TEYBE-	Tris-based extender + 20% eggyolk (EY) + 6% GLY (Non-centrifuged semen)
TEYBE+	Tris-based extender + 20% eggyolk (EY) + 6% GLY (Centrifuged semen)

Table 2. Post-thaw spermatological parameters of Honamli buck semen frozen with different semen diluents.

GROUP	MOTILITY(%)	PMAI(%)	HMMP(%)	MROS(%)	VIABILITY (%)
BIOXCELL	12.7±1.2^{c}	10.4±0.2^{b}	11.6±0.6^{b}	83.8±6.5^{a}	26.1±2.5^{c}
TLEBE-	58.5±1.9^{ab}	29.8±2.6^{a}	38.8±1.9^{a}	73.8±1.7^{ab}	61.2±1.9^{b}
TEYBE-	53.9±2.3^{b}	33.1±1.9^{a}	33.4±5.5^{a}	68.1±5.8^{ab}	55.9±1.8^{b}
TEYBE+	60.9±0.6^{a}	32.1±1.6^{a}	39.7±2.5^{a}	63.1±4.6^{b}	68.3±0.7^{a}
p	**	*	*	*	*

P<0.05 *, p<0.001 **,
$^{a.b.c.}$ The statistical significance is shown in the same column.
TLEBE-: Non-centrifuged Tris 1% Lecithin 6% Glycerol
TEYBE-: Non-centrifuged Tris 20% Egg yolk 6% Glycerol
TEYBE+: Centrifuged Tris 20% Egg yolk 6% Glycerol
PMAI: Plasma Membrane Integrity
HMMP: High mitochondrial membrane potential
MROS: Mitochondrial reactive oxygen species

Evaluation of In Vitro Spermatological Parameters

Motility

Semen motility (%) was assessed with a heated stage set to 37 °C and viewed at 400x magnification in the phase-contrast microscope (Inanc et al., 2023).

Evaluation of Flow Cytometric Analysis

Flow cytometric evaluation was conducted using a (Beckman Coulter) CytoFLEX flow cytometer, which had three channels with emission filters set at 610 ± 20 585 ± 42, 525 ± 40 nm, 488 nm blue laser. Around 10,000 events were analyzed per sample. Debris was eliminated by using the forward scattering area (FSC-A). All analyses were evaluated using software of CytExpert 2.3.

Preparation solutions were prepared using 0.153 mM JC-1 (Invitrogen, T3198), FITC-PNA 100 µg/ml (Sigma, L7381), Sybr-14 (1:10), propidium iodide (PI) (2.99 mM) (Invitrogen, L7011) and 5 µM MitoSOX (Invitrogen, M36008) dissolved in DMSO. The solution was then aliquoted into 50 µl portions and stored at -20 °C until needed. The plasma membrane, acrosome ingetrity (PMAI) in spermatazoa was assessed using a FITC-PNA/PI double staining protocol. Semen samples were extended with phosphate-buffered saline (PBS; 492 µl) to achieve a final concentration of 5×10^6 spermatozoa/ml. To this mixture, 5 µl FITC, 3 µl PI were added, bringing the final volume to 500 µl. After a 15-minute incubation in a 37 °C water bath in a dark environment. The FITC(-)/PI(-) population was identified as PMAI (Inanc et al., 2023).

Spermatozoa exhibiting high mitchondrial membrane potential (HMMP) were evaluated using the JC-1 protocol. Diluted sperm in PBS (495 µl) were to a final concentration of 5×10^6 spermatozoa/ml, followed by the addition of 5 µl JC-1 staining solution, bringing the total volume to 500 µl. The mixture was incubated for 15 minutes in a 37°C water bath in a dark environment before being analyzed by flow cytometry. HMMP levels were assessed (Inanc et al., 2023).

Spermatozoa viability was assessed using the Sybr-14 and PI double staining protocol. The sperm samples were extended in PBS (492 µl) to 5×10^6 spermatozoa/ml. Then, 5 µl Sybr-14 ,3 µl of PI were added to the semen mixture, was incubated for 15 minutes in a 37°C water bath in a dark environment before being analyzed by flow cytometry to evaluate sperm viability. Viability was determined as SYBR(-)/PI(+) population (Inanc et al., 2023).

Mitochondrial reactive oxygen species (MROS) in spermatozoa were assessed using the MitoSOX/PI double staining method. MitoSOX (5 µL) and PI (3 µL) were mixed with 492 µL PBS, and 10 µL of semen was added to the solution, bringing the sperm concentration to 5×10^6 spermatazoa/ml. The mixture was incubated at 15 minutes in a 37 °C water bath in a dark environment. The MITOSOX+/PI- population (%) was considered as mitochondrial ROS (Dönmez and İnanç, 2024).

Statistical Analysis

The Shapiro–Wilk test was performed to assess normality, and Levene's test was used to evaluate variances homogeneity. ANOVA identified group differences, followed by Duncan's test for post-hoc evaluation. The statistical analyses were evaluated 5% significance level, with a p-value of <0.05 indicating significance. The results are presented as means (X) ± standard deviations (SD), while non-parametric data are shown as means (X) ± standard error of the mean (SEM).

Results

The post-thaw spermatological parameters of Honamli bucks are shown in Table 2 based on the study's findings. The

findings revealed that the B extender group had the lowest motility, PMAI, HMMP, and viability rates after thawing, and these differences were found to be statistically significant ($p<0.001$). The TEYBE+ group exhibited the highest post-thaw viability rate in this study ($p<0.05$). Furthermore, the TEYBE- group demonstrated superior motility, PMAI, HMMP, and viability rates post-thaw when compared to the B group, although no advantage was observed relative to the TLEBE- and TEYBE+ groups. A comparison of MROS levels showed that the TEYBE+ group had significantly lower values than the B group ($p<0.05$).

Discussion and Conclusion

This study aimed to evaluate the efficacy of B, a commercial semen extender for bulls, in maintaining the quality of Honamli buck semen. Its performance was compared to Tris-lecithin and Tris-egg yolk-based diluents.

B extender is originally produced for bull semen, but the suitability of the diluent for buck semen has been questioned. Studies suggest that B extenders can notably enhance the motility and viability of sperm from bucks after freezing and thawing. In this study, the B group showed the lowest post-thaw sperm motility (12.7%) ($p<0.01$). Additionally, the B group exhibited the lowest PMAI value (10.4 ± 0.2%), the lowest HMMP value (11.6±0.6%) and the lowest viability (26.1 ± 2.5%) ($p<0.05$). Nethenzheni et al. (2021) reported post-thaw motility values for B-treated sperm as 68.2±13.5% for seminal plasma centrifuged samples and 85.0±3.4% for non-centrifuged samples. Moreover, a notable reduction in the percentage of viable and normal spermatozoa was found in the centrifuged B group (5.2 ± 4.9%) compared to the non-centrifuged B group (45.7 ± 21%). It was reported that the HMMP value for the non-centrifuged B group was 49.8±20.1%. Sarıözkan et al. (2010) reported that both centrifuged and non-centrifuged B groups gave better total motility than Tris groups. In the study by Emamverdi et al. (2015) the B group demonstrated a lower level of total motility in comparison to the tris-lecithin-based diluent group. However, no significant difference was found in contrast to the tris-egg yolk-based diluent group. This work also reported total motility as 47.6% and viability as 32.08% for the B group. Daşkın et al. (2011) reported the motility of 14 ± 2.2% and the plasma membrane integrity of 14 ± 3.4 for 200 × 10^6 spermatozoa per ml ratio dilution, the outcomes of this study support the findings observed in the present research. The reduced performance of B with Honamli buck spermatozoa may be attributed to their high content of polyunsaturated phospholipids, which increases susceptibility to oxidative stress. As a result, the glutathione levels in the B group were inadequate to shield the sperm from oxidative stress during freezing and thawing. The discrepancy in outcomes can be attributed to various factors, including the method of semen collection, season, and breed considerations, thereby generating additional inquiries concerning the suitability of B for ovine semen. Nonetheless, the considerable discrepancy between the studies is a matter of considerable interest.

The motility rates for the TEYBE- and TLEBE- groups were recorded as 53.9±2.3% and 58.5±1.9%, respectively in the present study. While the difference in motility between these groups was not statistically significant, the TLEBE- group showed a slight increase in motility. This evaluation is consistent with previous research indicating that the removal of seminal plasma from buck semen enhances motility in frozen-thawed sperm samples (Ferreira et al., 2014; Inanc et al., 2023; Sen and Tekin, 2015). The high content of unsaturated fats in buck spermatozoa membranes, combined with adverse interactions between sperm extenders and seminal plasma components, continues to hinder the success of cryopreservation. To minimize sperm toxicity caused by enzymes, such as egg yolk-coagulating enzymes, and specific proteins, it is recommended to remove seminal plasma via centrifugation when using extenders containing egg yolk or milk (Çevik and Tuncer, 2005; Inanc et al., 2023; Üstüner et al., 2020).

In Norduz goats, centrifugation of seminal plasma has been found to reduce sperm motility while increasing the percentage of abnormal spermatozoa (Sen and Tekin, 2015). Additionally, the characteristics of freshly collected semen, especially sperm motility and morphological integrity, are closely linked to post-thaw viability (Dorado et al., 2009). For instance, the addition of preservatives such as trehalose has been shown to enhance acrosome integrity and overall viability in cryopreserved ram semen (Zhao et al., 2020).The results from this study were comparable to the post-thaw values of Saanen breed buck semen frozen with skim milk containing 5% and 7% glycerol, as reported by Kulaksiz et al. (2013). Concurrently, the results showed thatthe post-thaw motility values of semen frozen by Büyükleblebici et al. (2014) using 6% glycerol in Ankara bucks were comparable to those observed in the present study. The present study revealed that the plasma membrane integrity values obtained from the TLEBE-, TEYBE-, and TEYBE+ groups (29.80%, 33.10%, and 32.10%, respectively) were significantly higher than those observed in group B (10.40%) ($P < 0.05$). The limited use of the B commercial extender in freezing studies restricts the evaluation of the results. However, similar results were achieved with the data obtained from lecithin and Tris egg yolk extender groups (Büyükleblebici et al., 2014; Emamverdi et al., 2015; Sharma and Sood, 2019; Üstüner et al., 2020), which indicated its similar effect on the preservation the plasma membrane structure. When the data on the viability rates of the study were evaluated, it was observed that the lowest value was obtained in group B (26.10%), while the TEYBE+ group (68.30%) was statistically higher than the TEYBE- (55.90%) and TLEBE- (61.20%) groups ($p<0.05$). The viability rates obtained by Sun et al. (2020) were determined to be 37.57% and 43.13% in the lecithin-containing groups and were found to be lower than those in present study. Although the values of our TEYBE- and control groups were similar, they were lower than those of the TEYBE+ group. The highest level of MROS was found in B (83.8±6.5%) ($p<0.05$). In addition, it was determined that the lowest MROS ratio was obtained in the TEYBE+ group, and the removal of seminal plasma

components from the environment increased the cryoprotective activity of the egg yolk.

Consequently, it was determined that the removal of seminal plasma in the extenders used for freezing buck semen had a beneficial impact on the freezing process, while the B extender was less effective in preserving buck semen compared to those containing lecithin and egg yolk. In light of the findings of this study and the discrepancies observed among the studies, it is not recommended to utilize B for freezing goat semen. Nevertheless, new studies are required to compare the effectiveness of commercial diluent in freezing buck semen.

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Conflict of Interest

The authors stated that they did not experience any real, potential or perceived conflict of interest.

Ethical approval

For this study, permission was obtained from Burdur Mehmet Akif Ersoy University Animal Experiments Local Ethics Committee with the number 07.12.2020/201-13.11.2024-1388. In addition, the authors declared that the Research and Publication Ethics were complied with.

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Author Contributions

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Data Collection and/or Processing: RÖ, ŞG
Analysis and/or Interpretation: FK, HAÇ, DK, MEİ
Literature Review: FM, MH
Manuscript Writing: FM, MH
Critical Review: AA

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