Effect of Egg Yolk and Removal of Seminal Fluid on Semen Cryopreservation in Norduz Goat

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Geliş tarihi: 28.09.2015 Kabul Tarihi: 09.11.2015

Abstract: Cryopreservation of goat spermatozoa is a valuable tool for genetic management. Previous studies have shown the frezeeablity of spermatozoa from various goat breeds but also have difficulties with available protocols. Therefore, we want to investigate the effects of centrifugation and different egg yolk ratios (5% and 10%) on spermatological parameters of Norduz goat spermatozoa. A total number of 20 ejaculates were collected with an artificial vagina from 5 mature Norduz male goats (3 and 4 years of age) twice a week, during the breeding season twice a week. Each ejaculate were divided into four equal aliquots. The samples were then diluted 1:9 (v:v) in washing solution, centrifuged at 600 g for 10 min at room temperature and the supernatant discarded for sperm washing process. Fresh and washed samples then were diluted with skim milk containing % 5 egg yolk and %10 egg yolk, respectively. Extended samples were loaded into 0.25 ml straws, equilibrated at that 4 C for 2h, frozen in nitrogen vapour for 10 min and plunged into liquid nitrogen. Samples were assessed for motility and acrosome integrity upon collection and after freezing and thawing. Removel of seminal fluid had detrimental effects on motility and percentage of total abnormal spermatozoa ($p<0,05$). In conclusion, it is not recommended to centrifuge the ejaculates form Norduz goats prior to conducting freezing-thawing procedures using a skim milk extender.
Keywords: *Acrosome, centrifugation, cryopreservation, egg yolk, goat semen*

Norduz Tekesi Spermasının Dondurulmasında Seminal Plazmanın Uzaklaştırılmasının ve Yumurta Sarısının İlavesinin Etkisi

Özet: Bu çalışma, santrifüj edilen ve santrifüj edilmeyen norduz teke spermasının farklı oranlarda yumurta sarısı içeren yağsız süt tozu sulandırıcısı ile sulandırılıp dondurulması ve dondurma sonrası spermatolojik özelliklerin saptanması amacıyla yapılmıştır. Çalışmada, 5 baş ergin Norduz tekesinden alınan ejakulatlar kullanıldı. Teklerden, çiftleştirme mevsiminde suni vagina kullanılarak haftada iki kez olmak üzere toplam 20 ejakulat toplandı. Alınan ejakulatlardan her biri 4 eşit hacme bölündü. Tüplerden ikisi seminal plazmanın uzaklaştırılmasını sağlayan santrifüj işlemi için yıkama solüsyonu ile sulandırılırken diğer 2 tüp santrifüj edilmedi. Yıkama işlemi için her bir ejakulat 1:9 oranında yıkama solüsyonuyla sulandırıldı ve oda ısında 600 g'de 10 dakika santrifüj edilerek süpernatant uzaklaştırıldı. Santrifüj edilen ve santrifüj edilmeyen sperma örnekleri sırasıyla % 5 ve % 10 oranında yumurta sarısı içeren yağsız süt tozu sulandırıcısıyla sulandırılarak 0.25'lik payetlere çekildi. Dondurma işlemi sıvı azot buharında gerçekleştirildi ve sıvı azot içinde saklandı. Çözdürme sonrası motilite ve total anormal spermatozoa oranı değerlendirildi. Seminal plazmayı ayırma işleminin motilite ve total anormal spermatozoa oranı üzerine zararlı etkisi olduğu gözlendi. Diğer yönden, % 10 yumurta sarısı motilite ve total anormal spermatozoa oranını olumlu yönden etkiledi ($p<0,05$). Sonuç olarak, aşım sezonu içinde % 10 yumurta sarısı içeren süt bazlı sulandırıcılarda seminal plazmanın uzaklaştırılmaması çözdürme sonrası sperma kalitesini artırdı.
Anahtar Kelimeler: *Akrozom, kreoprezervasyon, teke sperması, yumurta sarısı, santrifüj*

Introduction

Norduz goat is a domestic breed located in Eastern part of Turkey and take its name by breeders of the area due to its relatively high milk yield, resistance disease, preweaning viability and better growth performance (Daskiran et al., 2006). Norduz goat breed has reared a crucial role for milk, mohair production in the rural economy of eastern region and a mainly endangered genetic resource of Turkey (Ataseven et al., 2006). Genetic improvements of farm animals rely on intensive use of a few high quality males either for natural mating or for use in artificial insemination programs (Abdel-Rahman et al., 2000). Many countries have already started artificial insemination in goats, although its commercial application is not extensive (Qureshi et al., 2013). However, the main factor limiting more widespread use of frozen semen in caprine reproduction is the reduction of sperm viability during freezing processes (Batista et al., 2009). Successful cryopreservation depends upon several factors including cooling rate, thawing rate and addition of cryoprotectants (Fernandez-Santos et al., 2006). Seminal fluid most probably contains beneficial components to spermatozoa. However, the seminal plasma itself is not an optimal medium for sperm cooling and freezing (Brinsko et al., 2000). The most commonly used cryopreservation

diluents for goat semen have been either egg yolk or non-fat dried skim milk. However, goat sperm-freezing diluents containing egg yolk or milk can be harmful to the sperm cells (Ajadi et al., 2012). Egg yolk concentration is usually difficult to standardize in most cryopreservation protocols for semen in various species (Fernández-Santos et al., 2006). Especially at high concentration addition of egg yolk to semen extenders can be detrimental to spermatozoa (Santiago-Moreno et al., 2006). Negative interactions of the seminal fluid with the egg yolk in extender were first reported by Roy (1957). The seminal fluid were identified a protein (SBUIII) and an enzyme secreted named egg yolk coagulating enzyme (EYCE) by the bulbourethral glands, may cause toxicity to sperm in milk-based extenders (Purdy, 2006; Ferreira et al., 2014). The seminal fluid should be removed by centrifugation before sperm dilution in order to reduce the deleterious effects associated with bulbourethral gland secretion when egg yolk or milk containing extenders are used for storage of goat semen (Brinsko et al., 2000). But, centrifugation itself may cause both beneficial and damaging effects on sperm quality (Hoogewijs et al., 2010). Many studies determined that elimination of seminal fluid by centrifugation is necessary to increase post thaw motility and acrosomal integrity in goat semen, whereas other researchers reported better conclusions than the use of non-centrifuged goat semen in extender containing yolk when compared with centrifuged semen (Purdy, 2006; Ferreira et al., 2014). The aim of this study was to investigate the effects of seminal fluid and different of egg yolk (%5- %10) concentrations added to extender on post-thawed Norduz goat semen quality.

Materials and methods

Animals and semen collection: The study was conducted in breeding season, at education research and practise farm, faculty of Veterinary Medicine, University of Ankara. The animals were kept under natural light and maintained under and constant nutritional regime, with each buck being fed a daily diet of 1 kg concentrate, dried grass, salt lick and water ad libitum. Five mature norduz bucks (aged 3-4) with proven fertility were used and a total number of 30 ejaculates were collected with an artificial vagina, twice a week. Immediately after collection, the ejaculates were incubated in a water bath at 37°C, until microscopic sperm quality assessments were performed in the laboratory.

Semen evaluation: The ejaculates were evaluated and accepted for evaluation if the following criteria were met: volume varying between 0.75 - 2 ml; sperm concentration of 3×10^9 sperm/ml; the motile sperms percentage higher than 70% and less than 10% abnormal sperm in total. Sperm motility was assessed using a phase-contrast microscope (100x magnification), with a warm stage maintained at 37°C. 5 µl semen was placed directly on a microscope slide and covered by a cover slip. For each sample, at least five microscopic fields were examined by two trained observers. The mean of the two successive evaluations was recorded as the final motility score. For the sperm morphology assessment, at least 3 drops of semen were added to Eppendorf tubes, containing 1 ml of Hancock's (62.5 ml formalin, 150 ml sodium saline solution, 150 ml buffer solution, and 500 ml doubledistilled water) solution (Schafer and Holzmann, 2000). One drop of this mixture was put on a microscope slide and covered with a cover slip. The percentage of abnormal sperm (detached heads, acrosomal aberrations, abnormal mid-pieces and tail defects) was recorded by counting a total of 200 spermatozoa under phase contrast microscope (× 1000 magnification; oil immersion).

Semen processing: A total of thirty ejaculates were collected from 5 mature bucks and each sample was divided into four equal aliquots. Experiment was replicated six times. Two of them were diluted in the washing solution to remove the seminal fluid by centrifugation and two were kept as control. For washing procedure, the ejaculate was diluted 1:9 (v:v) in washing solution (250 mM Tris, 28 mM glucose, 104 mM citric acid, 0.05% streptomycin and 500 UI penicillin/ml) at 37 ºC, centrifuged at 600 g for 10 min at room temperature and the supernatant was discarded. And then, samples were diluted with skim milk (10 g skim milk powder and 0, 9 g glucose in 100 ml distilled water, heated to 95 ºC for 10 min), which contained 5% egg yolk and 10% egg yolk with a final semen concentration of approximately 2×10^8 sperm/mL, respectively. The diluted semen samples were then loaded into 0.25 mL French straws, sealed with polyvinyl alcohol powder and equilibrated at 5 ºC for 1 h. After equilibration the straws were frozen in liquid nitrogen vapor, (4cm above the liquid nitrogen), for 15 min and then plunged into liquid nitrogen for storage.

Statistical analysis: The mean post-thaw semen parameters of motility, dead spermatozoa, abnormal spermatozoa for the 10 trial carried out during this study were subjected to analysis of variance and differences among means were tested for significance by the Fisher's PLSD. The SPSS 10.0 software was used for all statistical analyses. Differences with values of $P<0.05$ were considered to be statistically significant.

Results

Progressive motility and acrosome membrane integrity of frozen–thawed semen diluted in 10% egg yolk were significantly higher than those diluted in 5% egg yolk (Tables 1) regardless of the effects of washing. When washing was not performed before freezing, progressive motility and acrosome membrane integrity were significantly higher than the other groups. Sperm motility values of extender containing 5% egg yolk were significantly lower in washed semen compared with the other groups. The percentage of abnormal sperm in extender containing 5% egg yolk were significantly higher compared with the othergrups.

Table 1. The effect of removal of seminal fluid and egg yolk level on sperm freezability of Norduz goats.

Egg yolk levels %	Centrifugation	Motility %	Abnormal Spermatozoa %
%5 (n=5)	+	48.4± 2.34^{d}	33.20±4.41^{a}
%5 (n=5)	-	59.0±2.45^{b}	25.70±3.42^{b}
%10 (n=5)	+	51.0±1.56^{c}	30.65±4.01^{c}
%10 (n=5)	-	65.65±2.80^{a}	22.65±3.70^{d}

Groups with different letters (a,b,c,d) in the same column are significant different (P<0.05).

Discussion and Conclusion

Chicken egg yolk has been traditionally used as additive for semen cryopreservation in several mammalian species (Santiago-Moreno et al., 2008). The advantageous of using egg yolk in frozen semen protect increase sperm resistance against cold shock and improve sperm survival (Gholami et al., 2012). Therefore, egg yolk has been used routinely in most semen cryopreservation protocols in domestic animals (Santiago-Moreno et al., 2006). Cryopreservation of goat semen with egg-yolk extender has been reported to have declining motility due to the presence of EYCE in the seminal fluid (Ajadi et al., 2012). For this reason, centrifugation process is applied to separate the sperm cells from the seminal fluid before freezing in order to overcome the negative interactions of seminal fluid after freezing and thawing (Castelo et al., 2010). High final concentrations of seminal fluid have been shown to be harmful to sperm cells during cooling and storage (Brinsko et al., 2000) due to activity of enzymes (Miro et al., 2009). Probably, the bulbourethral glands increase their activity by high plasma concentrations of prolactin and produce more phospholipase A enzyme (Ferreira et al., 2014) during the non-breeding season, since the chemical composition and volume of the ejaculate may be different depending on season (Ritar, 1993; Roca et al., 1992, Maxwell et al., 2007) The experimental procedure showed that lower egg yolk concentration (5%) does not appear to protect sperm against cryodamage by freeze–thawing regardless of centrifugation. On the other hand, egg yolk concentration above 10% in goat semen significantly increased the percentages of abnormal spermatozoa and motility compared to lower egg-yolk concentrations. In parallel to our study, remaining goat accessory gland secretions appear to be beneficial to preservation of sperm integrity (Pellicer-Rubio et al., 1997). Centrifugation process cause prominent changes in structural acrosome defects and negative effect on the sperm motility. But, several studies have reported that the washing procedure of goat semen for seminal fluid removal is necessary to increase motility, membrane integrity and fertility after freeze-thaw procedure (Kozdrowski et al., 2007; Machado and Simplicio, 1995). While several authors report that removal of the seminal fluid of chilled semen increases the sperm survival and motility (Miro et al., 2009; Kozdrowski et al., 2007; Machado and Simplicio, 1995), some other researches indicates positive results without washing (Arı and Daşkın, 2010, Cabrera et al., 2005; Daskin and Tekin, 1996). Even though there are several different results in the literature the underlying molecular basis is not yet clearly understood. This study demonstrates that the centrifugation procedure reduces the sperm motility and increases the morphological defects, after the freezing–thawing actions on the semen of Norduz goats during breeding season. This is a complex and time-consuming method that cause loss and damage to spermatozoa (Brinsko et al., 2000). In conclusion, it is recommended that a skim milk extender with 10% egg yolk should be used for non-washed semen during breeding season in Norduz goat. In addition to this, egg yolk ratio and centrifugation are important factors for successful cryopreservation of Norduz goat spermatozoa.

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