Effect of extenders on motility, morphology and osmotic resistance parameters of ram sperm during liquid storage

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SUMMARY

The aim of this study was to determine the effects of extenders in terms of motility, morphology and osmotic resistance (viability and HOS response: HE-test; modified hypoosmotic swelling test (HOST) associated with supravital eosin staining test) of diluted and liquid stored ram semen for seven days. A total of 5 Pırılak (Daglic × Kivircik, local breed) rams with satisfactory breeding potential were selected. Semen samples were collected by artificial vagina. Ejaculates were diluted to a final volume of 1/1 (semen/extender) with tris- (T), sodium citrate- (SC) and milk- (M) based egg-yolk extenders at room temperature and were stored at 4°C. The extender type had no effect on spermatozoa primary parameters, but extender type or time of storage had a significant effect on all final parameters. While sperm motility was gradually decreasing and the morphologically abnormal sperm rates gradually increasing, viability and HOST response changed during the storage period. Overall changes in all parameters over the storage period were less dramatic in semen diluted by T and SC comparing to semen diluted with M. In conclusion, the motility, morphology and osmotic resistance parameters of ram sperm declined, when storage period was increased and the magnitude of changes to parameters was less dramatic in semen diluted by T and SC extenders.

Keywords: Extender, HE-test, motility, morphology, ram semen.

RÉSUMÉ

Effet du milieu de dilution des spermatozoïdes de bélier sur la motilité, la morphologie et les paramètres de résistance osmotique du sperme au cours de leur conservation en milieu liquide

L’objectif de cette étude était de déterminer les effets de diluants en termes de motilité, de morphologie et de résistance osmotique du sperme (viabilité, HOS réponse et HE-test, test hypo-osmotique modifié de gonflement (HOST) associé au test de coloration à l’éosine) du sperme dilué et conservé en milieu liquide pendant 7 jours. Un total de 5 béliers Pırılak (Daglic × Kivircik, races locales) avec un potentiel de reproduction satisfaisant ont été sélectionnés. Les échantillons de sperme ont été collectés à l’aide d’un vagin artificiel. Les ejaculats ont été dilués (1/1, v/v) dans des milieux à base de jaune d’œuf contenant du tris- (T), du citrate de sodium citrate- (SC) ou du lait (M) et stockés à 4°C. Le type de diluant n’a pas eu d’effet sur les paramètres primaires des spermatozoïdes alors que les paramètres finaux ont été significa tivement affectés par le type de diluant et la durée du stockage. Alors que la motilité des spermatozoïdes a diminué progressivement et que le taux de spermatozoïdes anormaux a augmenté, la viabilité et la réponse au test HOST ont varié au cours du stockage. Globalement, les variations de l’ensemble des paramètres au cours de la période de stockage ont été moins importants pour le sperme dilué dans le milieu contenant du tris et ducitrate de sodium. En conclusion, la motilité, la morphologie et les paramètres de résistance osmotique du sperme de bélier sont affectés par la durée de conservation mais l’amplitude des variations des paramètres est moins importante lorsque le sperme est dilué en présence de tris ou de citrate de sodium.

Mots clés : Diluant, HE-test, motilité, morphologie, sperme de bélier.

Introduction

Semen dilution and storage are widely used in artificial insemination (AI) programs. Diluted and cooled ram semen is an alternative to frozen semen when the insemination is done within a short period of time after collection. Compared with fresh semen, cooled ram semen suffers from a decreased in motility and morphological integrity, accompanied by a decline in the survival in the female reproductive tract, reduction of fertility and increased embryonic loss (23). These damages are less pronounced in diluted and chilled semen than in frozen-thawed ram semen [1, 8].

Extenders differ in composition, depending on species, method, temperature of diluted semen storage, and the desired duration of storage [7, 11, 22, 33]. The aims of extenders are protection and maintenance of spermatozoa during processing and storage of the semen. Semen is usually diluted with Tris plus egg yolk, glucose phosphate solution, egg yolk–citrate solution, homogenized whole milk, fresh and dried skim milk, coconut milk, lactose solution and the commercial diluents [13, 37], and the some of them have been widely used in many farm animals, including ram. Comparing to natural breeding, the diluted and stored semen has low fertility rate, due to functional damage that it is not completely understood, but it is widely acceptable in the field.

One of the aims in the reproduction of domestic animals is to predict sperm fertilizing ability [30]. However, standard spermograms often do not provide reliable data in terms of ability to identify sub-fertile samples, so other tests have been developed. Osmotic tolerance has been described as a potential indicator of sperm function, which is used to predict fertility in the human and other mammalian species [4, 16, 18, 24, 30].

Revue Méd. Vét., 2011, 162, 11, 546-551
24, 31]. Thus PETRUNKINA et al. [29] have found a relationship between sperm-cell volume (measured electronically) in response to hypoosmotic changes and non-return rates within 56 days of the first insemination in bulls, and PÉREZ-LLANO et al. [28] have observed a relationship between hypoosmotic shock and fertility in boars. In studies about [13, 17, 20, 38] storage of ram semen in liquid form, the best effects were achieved at 4-5°C. Changes in motility during cooled storage period had been reported, according to the authors’ knowledge there has been no report evaluating the changes in motility and abnormal rate along with HE-test in ram sperm diluted with different extenders and stored at 4°C. Likewise, the HE-test was not screened in the previous studies. Therefore, the objective of the present study was to determine the changes in daily motility, morphology and osmotic resistance of Pirlak ram sperm that was diluted with Tris- (T), sodium citrate- (SC) and milk- (M) based egg-yolk extenders and stored at 4°C over a period of 7 days.

**Materials and Methods**

**SEmen collection and Processing**

Five sexually mature, 3 years old Pirlak (Daglic x Kivircik) rams are maintained at Afyon Kocatepe University, Research and Manipulation Farm of the Faculty of Veterinary Medicine in Afyonkarahisar, Turkey. Animals were fed by roughage and concentrate supplement and also received 500 g/day/head of concentrate mixture and 1.0 kg/day of dry alfalfa. During the breeding season, from each ram 5 samples of ejaculates were collected every other day using an artificial vagina. Immediately after collection, the ejaculates were immersed in a warm water bath at 37ºC until their assessment in the laboratory. Semen assessment was performed in approximately within 10 minutes after collection. All sperm parameters in the ejaculate were correct (motility ≥ 80%; total morphology abnormalities < 10%). The collected semen was mixed 5 times. Semen concentration was split into three equal fractions and diluted in 1:1 (v/v) rate with extenders and stored at 4°C. Likewise, the HE-test was not screened in the previous studies. Therefore, the objective of the present study was to determine the changes in daily motility, morphology and osmotic resistance of Pirlak ram sperm that was diluted with Tris- (T), sodium citrate- (SC) and milk- (M) based egg-yolk extenders and stored at 4°C over a period of 7 days.

**Extendeders**

Three extenders (T, SC and M) were used in the present study. All chemicals were purchased from Sigma Chemical Co. (Interlab Ltd., Eskisehir, Turkey). The extenders were prepared as follows:

*Extender T:* Tris-based egg-yolk extender was a solution containing Tris (3.63 g), fructose (0.5 g); citric acid (1.99 g), 100 ml and mixed with 15 % (v/v) of egg yolk.

*Extender SC:* Sodium citrate-based egg-yolk extender was prepared from a 2.9% aqueous solution of trisodium citrate and supplemented with 20 % (v/v) of egg yolk.

*Extender M:* Milk-based extender was prepared out of non-fatty milk powder (11 % (w/v)) and distilled water, heated to 95°C for 10 min., and then cooled to room temperature before the egg yolk was added (5 % (v/v)).

**Stored Semen Evaluation**

After the primary parameters evaluation, the samples in water bath were stored at 4°C in one step. Every 24 hours, for whole period of study (7 days), all samples which were kept in 4°C were tested for sperm motility, total morphological abnormalities and HE-test. Sperm motility was subjectively estimated according to the standard method [2]. Using a phase contrast microscope (Olympus CX31, Olympus Optical Co., Ltd., Japan) magnification at 400 × equipped with a heated stage adjusted to 37°C. Motility estimations of each sample were performed in five different fields by the same person throughout the study. The mean value averaged from five successive estimations was used as the final motility score. The proportion of morphologically abnormal sperm cells was estimated by wet mount slide using two to three drops of semen diluted in Hancock’s solution [14]. A drop of this mixture was placed on a slide and covered with a cover slip. The percentage of total spermatozoa abnormality (acrosomal abnormality, detached heads, abnormal mid-pieces and tail defects) was determined by counting a total of 400 spermatozoa under the phase contrast microscope (magnification 1000 ×, oil immersion).

The osmotic resistance was evaluated by a modified cumulative analysis of hypoosmotic staining test (HE-test), using the eosine exclusion test and the Hypoosmotic Swelling Test (HOST) [6,10,21]. Semen samples were diluted 1:10 (v/v) in 100 mOsm fructose solution including 1% (w/v) eosin-Y, and were incubated in a water bath at 35°C for 30 minutes. The sperm suspension smears were prepared with 10 µL of mixed samples, and 100 sperm cells were observed in each slide under a phase contrast microscope at 400× magnification and they were classified into four types (Type I: tail swollen and head white, HOS+/E-; Type II: tail non-swollen and head white, HOS-/E-; Type III: tail swollen and head red, HOS+/E+; Type IV: tail non-swollen and head red, HOS-/E+) according to staining status of sperm head and curling of the sperm tail in smears.

**Statistical Analysis**

All statistical analyses were performed using the Statistica® package program, version 6.0 (Statsoft Inc., Tulsa, OK, USA). Mean values were evaluated by analysis of variance (ANOVA). Tukey’s post hoc test was used to compare the significance of the differences among three extender groups in terms of the effects on the sperm motility, abnormal sperm rate and HE-test features based on the storage period. Pearson’s correlation coefficients were used to evaluate the correlations among the parameters. Results were expressed as mean ± standard error of the mean (S.E.M.). Differences were considered as statistically significant at the $P<0.05$ level.

**Results**

Before the storage at 4°C, parameters were estimated and saved as primary (Day 0). The mean primary parameters of the sperm motility, abnormal sperm rate and HE-test were
Discussion

This study investigated the effect of extender on sperm motility, morphology and osmotic resistance parameters stored at 4°C over a period of 7 days. The sperm quality is considered a valuable and reliable measure of male fertility estimation. Normal spermatzoa lose fertilizing ability before they lose motility. Furthermore, normal spermatzoa may exhibit normal movement and yet be incapable of fertilizing an egg. In addition, live-dead sperm proportions are highly correlated with visual estimates of progressively motile spermatzoa. It is well known that normal sperm counts and other variables measured in routine semen analysis do not ensure good fertility rate. The integrity of caudal plasma membranes of spermatzoa exposed to hyposmotic swelling, HOS test, assessed the resistance of the sperm to damage induced by the loss of permeability under the stress of swelling driven by the hypoosmotic treatment. The results of previous studies indicate that an intact sperm cell membrane will more closely reflect semen fertility than sperm motility [1, 3, 9, 16, 27]. HE-test may have more to offer than straight viability testing in that the HOST, fertility than sperm motility [1, 3, 9, 16, 27]. HE-test may have more to offer than straight viability testing in that the HOST, fertility than sperm motility [1, 3, 9, 16, 27]. HE-test may have more to offer than straight viability testing in that the HOST, fertility than sperm motility [1, 3, 9, 16, 27]. HE-test may have more to offer than straight viability testing in that the HOST, fertility than sperm motility [1, 3, 9, 16, 27]. HE-test may have more to offer than straight viability testing in that the HOST, fertility than sperm motility [1, 3, 9, 16, 27]. HE-test may have more to offer than straight viability testing in that the HOST, fertility than sperm motility [1, 3, 9, 16, 27]. HE-test may have more to offer than straight viability testing in that the HOST, fertility than sperm motility [1, 3, 9, 16, 27]. HE-test may have more to offer than straight viability testing in that the HOST, fertility than sperm motility [1, 3, 9, 16, 27]. HE-test may have more to offer than straight viability testing in that the HOST, fertili...
EFFECT OF EXTENDERS ON RAM SPERM PARAMETERS

The possible physiological reasons for this decline might be effects of seminal plasma constituents and endogenous free radical production. Cellular metabolism is not completely gone during liquid storage. PAULENZ et al. [26] reported better sperm motility and membrane integrity in tris-based extender than both the sodium citrate- and milk-based extenders for liquid storage semen. In addition, LOPEZ et al. [19] observed that sodium citrate-based extenders maintained sperm motility longer than milk-based extenders. There is a gradual decrease in motility and morphological integrity, and a rapid decrease in fertility [15, 23]. Contradictory results were reported for frozen-thawed semen by GIL et al. [12] found significantly higher percentages of uncapacitated spermatozoa in milk extender than in tris-citrate-fructose extender. It was reported that organic peroxides are produced when ram semen is held at 5°C and that this accumulation is related to the sperm quality loss [17, 19, 26]. MAXWELL and STOJANOV [22] added some antioxidants to diluent Tris-glucose-egg yolk 20% (v/v) for liquid storage, and these antioxidants improved both survival and acrosome integrity of sperm during liquid storage at 5°C, although the beneficial effects of the antioxidants were not maintained throughout the 12 days of storage. These differences affected their role in countering oxidative stress and possibly played a role in the daily reduction of sperm structural and functional parameters. Comparison of the differences between extenders and daily values of sperm motility, morphology and osmotic resistance parameters were tested to estimate changes during the storage period. This revealed that there were significant changes from Day 1 onwards. The differences between both storage period and extenders might be due to contents of diluters especially the glucose and egg yolk lipoproteins may have positive effects on the sperm. However, the decline in the parameters during storage period may be due to run out the energy source of sperm and the sperm quality decrease may originate from the metabolic activity under 4°C since the pH is changed by the metabolic products resulting in intoxication. Storage at 4°C does not completely arrest spermatozoa metabolism; therefore, the accumulation of the toxic products, including free radicals, might be involved in the damage suffered by spermatozoa. The sperm plasma membrane is rich in polyunsaturated fatty acids and is therefore susceptible to pe-

Figure 3: Effects of extenders on HE-test of ram sperm during storage at 4°C for 7 days. Data are means ± S.E.M. (T: Tris-based, SC: Sodium citrate-based, M: Milk-based egg yolk extenders). A-C: The different uppercase letters are significant among extenders within days of storage; a-f: The different lowercase letters are significant among days of storage within extenders. HOS+/E-: tail swollen and head white; HOS-/E-: tail non-swollen and head white; HOS+/E+: tail swollen and head red; HOS-/E+: tail non-swollen and head red.

Revue Méd. Vét., 2011, 162, 11, 546-551
The results of this study indicated a clear advantage of using the tris-based extender instead of sodium citrate-based and milk-based extenders for storage of liquid ram semen. In particular, ram semen diluted and stored in tris-based extender maintained sperm quality during a longer period of time. The > 50% motility was observed through 4 days especially in semen diluted with T and SC extenders and the daily motility loss was high in M diluent (Figure 1), but the alive and HOS response was observed higher than 50% through 3 days especially in semen diluted with T extender and daily sperm viability and membrane integrity degradation in this extender was minimum (Figure 3). However, the difference between T and SC extenders was not found for morphologically abnormal sperm rates just after dilution for 4 days (Figure 2). Therefore, Tris- and sodium citrate-based extenders were more suitable to ram semen as diluters than milk-based extender. Nevertheless, milk-based extender may be used as extender within two days for dilution alternatively when needed.

In conclusion, motility, morphology and osmotic resistance parameters of ram sperm declined with the days of storage. The changes in the motility, morphology and osmotic resistance parameters over the storage period were less dramatic than the sodium citrate-based and milk-based extenders, respectively.

References

EFFECT OF EXTENDERS ON RAM SPERM PARAMETERS


