Slov Vet Res 2012; 49 (2): 97-102
UDC 636.3.082.4:612.616.2:57.086.13

Original Scientific Article

THE EFFECT OF DIFFERENT DILUTION RATES ON POST-THAW QUALITY OF RAM SEMEN FROZEN IN TWO DIFFERENT EGG-YOLK FREE EXTENDERS

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Summary: The objective of the present study was to evaluate the effects of pre-freezing sperm concentration (200, 400 or 800x10^6 spermatozoa/ml) using two commercial extenders (Bioexcell® and Andromed®) on post-thaw survival and acrosomal status of ram spermatozoa. Semen samples were obtained from the 5 mature Karayaka rams (aged 2-3 yr) and a total of 30 ejaculates collected from each male twice a week for 3 weeks with the aid of an artificial vagina, during the non-breeding season (February, winter). Semen extended in Bioexcell® or Andromed® diluents, was loaded into 0.25 ml straws and equilibrated at 4°C for 2 h. Straws were frozen in the vapour of liquid nitrogen and then stored at -196°C. After thawing (at 37 °C for 30 sec), sperm motility, acrosomal status and membrane integrity were assessed. Pre-freezing sperm concentration influenced ($P<0.001$) frezability of spermatozoa and affected all the in vitro parameters at 400x10^6 and 800x10^6 spermatozoa/ml negatively regardless of the extender. Decreasing the sperm concentration into 200x10^6 spermatozoa/ml influenced positively the percentage of sperm motility and membrane integrity extended in Bioexcell® (40 %, 29 %) and Andromed® (43 %, 32 %). The lowest percentage of abnormal acrosome was also described at lowest sperm concentration (200x10^6 spermatozoa/ml) in both extenders as 29 and 26 %. It was concluded that significant differences exist between the dilution rates or sperm concentrations. Lower sperm concentrations or higher dilution rates with the commercial extenders were better to protect sperm from damages during cryopreservation.

Key words: ram; semen; dilution rate; commercial extender; cryopreservation

Introduction

Egg yolk is a main component in the extenders for storage and cryopreservation of semen in most mammalian species including bull, ram, goat. The main effective component of egg yolk is the low density lipoprotein fraction like lecithin, which protects the membrane phospholipid integrity during cryopreservation (1). However, in recent years, there has been frequent opinion against the use of egg yolk due to the wide variability of its constituents, which makes evaluation of its beneficial component complex. Furthermore, egg yolk increases the risk of microbial contamination and thereby allows subsequent production of endotoxin, which may reduce after thawing viability and acrosome integrity of spermatozoa in some species such as ram, goat, and buffalo (2-8). Therefore, it would be preferable to use the diluents free from egg yolk. Andromed® and Bioexcell® is a commercially available extender without components of animal origin. It contains vegetal lecithin as a cold shock protector, and has been used for freezing buck and ram semen with satisfactory results (9, 10, 11).

The survival of frozen–thawed ram sperm is affected by many factors, extensively reviewed by Salamon and Maxwell (12). The rate of semen dilution is one factor determining cryopreservation

Received: 6 January 2012
Accepted for publication: 3 April 2012

success but there are few comparative studies on the effects of sperm concentration for freezing ram semen (13). The rate of dilution is usually varied to produce a standardized number of spermatozoa per inseminate dose or is simply based on the number of females to be inseminated per ejaculate (12). Early researchers employed dilution rates ranging from 2 to 5 fold, which are significantly higher than the 10–15 fold dilution rates commonly used today (12). Excessive dilution has been reported to cause membrane destabilization and capacitation-like changes in spermatozoa and cryopreservation may have an additive effect, further injuring the cells. It is thought the "dilution effect" is due to the removal of protective factors in seminal plasma (14). However, there is a lack of information on the pre-freezing rate to which spermatozoa can be diluted without a reduction in their post-thaw survival. Moreover, there are no studies concerning the effect of Bioxcell® and Andromed® extenders on ram semen freezability.

The aim of the present study was to evaluate the effects of three different pre-freezing dilution rates on sperm motility, acrosome abnormality, and plasma membrane integrity, in Karayaka ram semen, frozen in egg yolk free commercial extenders, Andromed® and Bioexcell®.

Materials and methods

Animals and semen collection

Five mature Karayaka rams (aged 2-3 years) with proven fertility were housed at the Education Research and Practice Farm, Faculty of Veterinary Medicine, University of Ankara, Turkey at 39 ° 57′ N, 32 ° 53′ E, at an altitude of 850 m. The rams (65-70 kg) were kept under natural light and maintained under a uniform and constant nutrition regime with each ram being fed a daily diet of 1 kg concentrate, dried grass, salt lick and water ad libitum. A total number of 30 ejaculates were collected using artificial vagina from each male twice a week for 3 weeks during the nonbreeding season (February, winter).

Semen extenders

Two extenders, Bioxcell® (Extender B) and Andromed® (Extender A), were used for the dilution of semen in the present study. The extenders were prepared as follows:

Extender B: A commercially available diluent (IMV, Aigle, France). This extender contains soybean extract with antibiotics (lincomycin, spectinomycin, tylosin, gentamycin) and glycerol (7%).

Extender A: A commercially available diluent (Minitüb, Tiefenbach, Germany). This extender contains: bi-distilled water, fructose, glycerol, citric acide, buffers and phospholipids with antibiotics (lincomycin, spectinomycin, tylosin, gentamycin) and glycerol (6.7%).

Semen dilution, freezing and thawing

Only ejaculates with a minimum concentration of $3x10^9$ spermatozoa/mL and 70% progressively motile cells were pooled across rams. The volume of semen ejaculates were measured in a conical tube graduated at 0.1 ml intervals and the sperm concentration was estimated using a haemocytometer (15). Semen extended Bioexcell® or Andromed® in diluent to a final concentration of 200, 400 or $800x10^6$ spermatozoa/ml was loaded into 0.25 ml plastic straws (IMV, Laigle, F-61300, France) and sealed with polyvinyl alcohol (PVA). Straws were equilibrated at 4 °C for 2 hr and after equilibration, the straws were suspended on a styrofoam rack 4 cm above the liquid nitrogen (vapour) for 15 min. The straws were plunged into the liquid nitrogen; where stored until thawing. After storage for a period of 3 weeks, the semen straws were thawed in a water bath (37 °C for 30 s) for microscopic semen evaluation immediately after thawing. The experiment was conducted in six replicates.

Semen evaluation

Sperm motility was evaluated subjectively using a phase-contrast microscope (400x), with a warm stage maintained at 37°C. A wet semen mount was made using 5 µL semen placed directly on a microscope slide and covered by a cover slip. For each sample, at least 5 microscopic fields were examined by 3 trained observers. The mean of the three successive evaluations was recorded as the final motility score (5).

For the assessment of acrosomal abnormalities, at least three drops of each sample were added to an Eppendorf container containing 1 mL Hancock solution (62,5 mL formalin, 37%), 150 mL saline solution, 150 mL buffer solution and 500 mL double-distilled water) (16). One drop of this

semen mixture was put on a slide and covered with a cover slip. The percentage of the acrosomal abnormalities was determined by counting a total of 200 sperm under phase-contrast microscope using an immersion objective.

The hypo-osmotic swelling test (HOST) was used to evaluate the functional integrity of the sperm membrane, based on curled and swollen tails, by incubating 100 µL semen with 1000 µL of a 100 mOsm hypo-osmotic solution (9 g fructose and 4.9 g sodium citrate per liter of distilled water) at 37°C for 30 min. After incubation, 5 µL of the mixture was spreaded with a cover slip on a warm slide. A total of 200 spermatozoa were counted in different fields at 400x under phase contrast microscope and percentage of spermatozoa positive to HOS test (having coiled tails) was determined (17).

Statistical analysis

The study was repeated 6 times and the results were expressed as mean±SEM. One-way analysis of variance (ANOVA) with a subsequent Tukey's test was used to compare the mean values resulting from the various treatments at a significance level of $p < 0.05$. All analyses were carried out using the SPSS 11 for Windows statistical software package.

Results

The effects of three different pre-freezing dilution rates on sperm motility, acrosome abnormality, and plasma membrane integrity, in Karayaka ram semen frozen in egg yolk free commercial extenders, Andromed® and Bioexcell® are presented in Table 1. Pre-freezing sperm concentration influenced ($p<0.05$) freezability of spermatozoa and affected negatively all the *in vitro* parameters at $400x10^6$ and $800x10^6$ spermatozoa/ml dilution rates regardless the extender type. The decrease of sperm concentration to $200x10^6$ spermatozoa/ml influenced positively the percentage of motility and the membrane integrity; extended in both commercial extenders. The proportion of spermatozoa with intact acrosome was influenced by either pre-freezing sperm concentration or extender. The highest proportion of spermatozoa with intact acrosomes were observed in samples frozen with Bioxcell® and Andromed® at the $200x10^6$ spermatozoa/ml compared to $400x10^6$ and $800x10^6$ spermatozoa/ml.

Discussion

These results demonstrate that there is a considerable reduction in ram sperm motility, acrosome and membrane functionality following cryopreservation at low sperm dilution rates or with increased numbers of spermatozoa per dose. Conforming the results of similar studies (13, 18).

A commercially available extender in which egg yolk is substituted by soybean lecithin has been recently tested in different species (19, 20, 21). Fukui et al. (9) reported that Andromed® rendered fertility results comparable to egg yolk extenders, after intrauterine insemination of sheeps. This makes Andromed® a promising option for further research on sheeps. And also Bioexcell® is recommended due to its animal ingredient free properties. It was developed for bovine semen cryopreservation and has been applied to other animal species including

Table 1: Effect of different dilution rates on motility, acrosomal abnormality and plasma membrane integrity after thawing (n=6)

Extender	Sperm Concentration ($x10^6$sperm/ml)	Motility (%)	Acrosomal abnormality(%)	Plasma membran integrity (%)
Bioxcell®	200	40±3.5a	29±2.1a	29± 3.1a
	400	20±1.5b	40±2.5b	18±1.9b
	800	13±1.2b	48±2.6b	14±2.0b
Andromed®	200	43±4.5a	26±3.5a	32±2.8a
	400	30±2.5b	34±2.0b	22±1.7b
	800	21±2.8b	44±2.3b	19±1.4b

a,b: Different superscripts in the same column indicate significant differences ($p<0.05$).

sheep and goat (10, 11). Gil et al. (10) reported that ram spermatozoa in soya lecithin based extender Bioxcell® maintained the sperm quality and produced acceptable fertility rates.

One of the fundamental steps of semen manipulation is described by the dilution rate according to a constant concentration of spermatozoa in the current study. Post-thawing characteristics of spermatozoa were significantly impaired by increase of prefreezing sperm concentration to 400 and 800x10^6 spermatozoa/mL.

D'Alessandro et al. (13) also found a positive effect of high pre-freeze extension of ram spermatozoa, where the highest post-thaw motility and acrosome integrity was observed when semen was diluted to 200 million spermatozoa per ml and lowest after dilution to 800 million per ml. These results agree with the idea of better post-thaw seminal characteristics in lower sperm concentrations. Samper and Morris (22) suggested that freezing at lower sperm concentrations may initially provide a higher availability of nutrients and increase cryoprotectant property per spermatozoon, which may explain the higher percentage of motile spermatozoa immediately after thawing when frozen at 100x10^6 ml. In parallel, Leahy et al. (18) reported that ram spermatozoa may be extended at high rates prior to freezing, at least to 20 million cells per mL, but not post-thaw. Dilution prior to freezing improved motility, viability and acrosome integrity when assessed over 6 h of incubation period.

A dose of 25–50x10^6 sperm for laparoscopic intrauterine insemination, 75-100x10^6 sperm for transcervical insemination and 150-300x10^6 for cervical insemination is recommended in ewes (23). In the present study, three concentrations (200x10^6, 400x10^6 and 800x10^6) of spermatozoa were selected to assess the changes after thawing. The changes in the sperm structure function and motility parameters were less dramatic in the 200x10^6 ml compared to 800x10^6 and 400x10^6 ml. The possible physiological reasons for the decline might be due to extracellular oxidative stress, effects of seminal plasma volume-constituents and endogenous free radical production. Substances from seminal plasma protect spermatozoa from premature aging during storage (24). It is suggested that the amount of seminal plasma surrounding each spermatozoon in an ejaculate varies among different concentrations (25). Along with this fact, the higher volume of extender and its contents may be one of the reasons for better preservation of functional and motility parameters at the lower concentration in our study. In this way, lower sperm concentration increased the percentage of intact plasma membrane and acrosome integrity, similar to the effect on post-thaw seminal characteristics. Possibly the frozen spermatozoa in 200x10^6 ml had a higher ratio of cryoprotectant agents per cell than 400x10^6 ml and 800x10^6 ml at a higher sperm concentration. The higher the amount of cryoprotectant per sperm cell, possibly the higher the percentage of unfrozen water channels, leading to better post-thaw seminal characteristics (26).

To conclude, commercial extenders seems to be useful as an alternative to the conventional extenders (Tris based, Milk Based) at higher dilution rates for the freezing of ram semen. The results of this study only reveal that these two extenders are suitable for cryopreservation of ram semen for in vitro use. In order to evaluate the use of the extenders for in vivo use, further studies are necessary.

Acknowledgements

This study was supported by grants from TUBİTAK, Turkey (KAMAG-106G005). The authors wish to thank Aykut Üner for statistical assistance.

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VPLIV KONCENTRACIJE OVNOVEGA SEMENA NA NJEGOVO KAKOVOST PO ZAMRZOVANJU V DVEH RAZLIČNIH RAZREDČEVALCIH BREZ JAJČNEGA RUMENJAKA

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Povzetek: Namen dela je bil oceniti vpliv koncentracije ovnovega semena pred zamrzovanjem (200, 400 oz. 800 milijonov semenčic/ml) z uporabo dveh komercialnih razredčevalcev (Bioexcell ® in Andromed ®) na preživetje semenčic in stanje njihovega akrosoma. Vzorci semena so bili pridobljeni od 5 spolno zrelih ovnov pasme karajaka, starih 2-3 leta. Skupno je bilo s pomočjo umetne nožnice odvzetih 30 ejakulatov. Odvzem smo opravili v treh tednih v neparitvenem obdobju (februar), vsakemu ovnu pa smo seme odvzeli dvakrat tedensko. Seme smo razredčili z razredčevalcem Bioexcell ® ali Andromed ® in ga spravili v 0,25 ml slamice za zamrzovanje. Najprej smo ga ohladili na 4 °C za 2 uri, nato pa ga zamrznili v pari tekočega dušika in ga shranili pri -196 °C. Po odmrzovanju (pri 37 °C za 30 sekund) smo ocenili gibljivost semenčic, stanje akrosoma in celovitost membrane. Ugotovili smo, da koncentracija semena pred zamrzovanjem vpliva na uspešnost zaščite semenčic ($p < 0,001$). Koncentraciji 400 milijonov in 800 milijonov semenčic/ml sta neodvisno od razredčevalca negativno vplivali na preživetje semenčic. Zmanjšanje koncentracije semena na 200 milijonov semenčic/ml je pozitivno vplivalo na gibljivost semenčic in celovitost membrane v razredčevalcih Bioexcell ® (40 %, 29 %) in Andromed ® (43 %, 32 %). Pri najnižji koncentraciji semena (200 milijonov semenčic/ml) smo neodvisno od razredčevalca opazili tudi najnižji odstotek nenormalnih akrosomov (29 % in 26 %). Iz naših rezultatov lahko povzamemo, da so semenčice bolj zaščitene pred poškodbami med zamrzovanjem pri nižjih koncentracijah semena oz. višji stopnji redčenja s komercialnimi razredčevalci.

Ključne besede: oven; redčenje; komercialni razredčevalci; zaščita med zamrzovanjem