Effect of age on Boujaâd ram semen quality extended in skim milk and tris egg yolk at 5°C

A. BADI[1,2], A. BEN MOULA[1], K. EL KHALIL[1,2], B. NASSER[2], B. EL AMIRI[1]

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Abstract

The aim of the present work was to study the effect of age (2.5 years vs 6 years) and extender (skim milk vs tris egg yolk) on the storage of liquid semen at 5°C. Semen was collected weekly from Boujaâd rams and extended to a final concentration of 0.8 x 10^9 spermatozoa/ml. Sperm quality assessment included total and progressive motility, viability, morphology, membrane integrity and lipid peroxidation assay. Sperm quality was assessed at 0, 8 and 24 hours of storage. Sperm quality decreased over time. There was a significant interaction between age, storage time and extender for progressive motility. Older rams had the significantly lower quality of sperm after storage. Skim milk preserved motility better than Tris-egg yolk. While Tris-egg yolk preserved membrane integrity and inhibited lipid peroxidation. In conclusion, the age of rams should be taken into account for artificial insemination programs using liquid stored semen. Also, type of extender should be selected based on the desired effect.

Keywords: Ram semen, extender, liquid storage, Boujaâd, Age.

Effet de l'âge sur la qualité du sperme de bélier de la race Boujaâd conservé dans le lait écrémé et dans le Tris jaune d'œuf à 5°C

Résumé

Le but de ce travail était d'étudier l'effet de l'âge (2,5 ans vs 6 ans) et de diluant (lait écrémé vs tris jaune d'œuf) sur le stockage du sperme à l'état liquide à 5 ° C. Le sperme de béliers Boujaâd a été prélevé chaque semaine puis conservé à une concentration finale de 0,8 x 10^9 spermatozoïdes/ml. L'évaluation de la qualité du sperme pendant le stockage comprenait la motilité totale et progressive, la viabilité, la morphologie, l'intégrité de la membrane et la peroxydation lipidique. La qualité du sperme a été évaluée à 0, 8 et 24 heures de stockage La qualité du sperme a diminué avec le temps. Il y avait une interaction significative entre l'âge, le temps de stockage et le diluant pour la motilité progressive. Le sperme de béliers plus âgés avait une qualité nettement inférieure après le stockage. Le lait écrémé garde mieux la motilité par apport au Tris jaune d'œuf. Alors que le Tris jaune d'œuf préserve mieux l'intégrité de la membrane et diminue la peroxydation lipidique. En conclusion, l'âge des béliers devrait être pris en compte pour les programmes d'insémination artificielle utilisant le sperme stocké à l'état liquide. De plus, le type de dilueur doit être sélectionné en fonction de l'effet souhaité.

Mots-clés: Sperme de bélier, diluant, stockage liquide, Boujaâd, âge

INTRODUCTION

The Boujaâd is one of the five most important Moroccan sheep breed reared in arid and semi-arid areas due to its important production attributes (Boujenane *et al*., 1995; Chikhi and Boujenane, 2003). It is considered a seasonal breed, with the peak period of reproduction in summer and autumn. The breed is limited to the Bejaâd region of Morocco.

Artificial insemination with stored liquid semen would be a great asset to expand the use of valuable rams and diversify the genetics. Age of ram and type of semen extender are factors that could influence sperm preservation (Paulenz *et al.,* 2002; Hassan, 2009; Tabba *et al.*, 2006; Focşăneanu, 2014). Several extenders have been used for ram semen storage of these Tris-egg yolk and skim milk extenders remain the most widely used. These extenders are easy to prepare, economical and result in acceptable conception rate in artificial insemination programs (Maxwell and Salamon, 1993; Leboeuf, 2000). It is well established that extenders protect sperm during preservation through different mechanisms. Milk-based extenders provide this protection mostly through casein micelles (the major proteins of milk) (Flipse, 1954; Salamon and Maxwell, 2000). While egg yolk-based extenders provide this protection through the low-density lipoproteins (LDL) (Farshad and Holtz, 1994; Kasimanickam, 2011; Paulenz, 2002).

Sperm production and quality increase with age after puberty (Tabbaa *et al*., 2006; Štolc *et al.*, 2009; Focşăneanu *et al.,* 2014), then plateaus around 3 years of age (Hassan *et al.*, 2009). Several studies have shown a significant decrease in ejaculate quality in aged rams (Chella *et al.*, 2017; Štolc *et al*., 2009). This decrease in sperm quality with age has been attributed to the onset of testicular de-

[1] INRA-Centre Régional de la Recherche Agronomique de Settat, BP589, Settat, Morocco. Correspondence: bouchraelamiri@yahoo.fr

[2] Laboratoire de Biochimie et Neurosciences, Faculté des Sciences et Techniques, Université Hassan 1er, BP. 577, 26000 Settat, Morocco

generative changes. In breeds with small populations, such as Boujaâd, rams tend to be maintained in reproduction for a longer time because of the lack of availability of a replacement. The present study was designed to compare quality of sperm from young adult rams (2.5 years) and old rams (6 years) after liquid storage at 5°C in the two most commonly used extenders (Skim milk vs Tris-egg-yolk).

MATERIAL AND METHOD

Chemicals

Unless otherwise specified, all chemicals used in this study were purchased from Sigma (St.Louis, MO, USA) and Merck (Merck Schuchardt OHG, Germany).

Animals and samples collection

Two groups of mature and fertile Boujaâd rams, Young M: (2.5-3 years of age; n= 5) and aged A: (6 years of age; n= 3) were used in this study. The rams were housed at the laboratory of Reproductive Biology, INRA-Morocco, Regional Center of Settat (32° latitudes). Animals were clinically healthy and did not show any abnormalities of the external genitalia. All animals were kept indoors in a free large covered shelter. Rams were fed a diet of straw–hay, and barley and sunflower as a concentrate. Daily amounts provided per head were 1000 g of hay and 1200 g of concentrate given in two equal quantities and a free access to water. Ejaculates (6 ejaculates per ram) were collected using an artificial vagina (40-42°C) during the breeding season. Immediately after collection, ejaculates were placed in a water-bath at 37°C. Sperm concentration was evaluated using a calibrated spectrophotometer (Photometer SDM6, Minitube, Germany). Only ejaculated with concentration > 2 x 10^9sperm/ml, mass motility > 3, and individual motility > 70% were used in the experiment.

Extender preparation and semen storage

Two extenders (Skim milk and Tris-egg-yolk) were used. Skim milk extender consisted of 11g skim milk in 100 ml distilled water heated 10 min at 95°C (Colas *et al*.,1968). Tris egg yolk consisted of 2.666 g Tris, 0.44 g fructose, 1.398 g citric acid, egg yolk 12% in 100 ml distilled water and adjusted to pH 6.8. Penicillin and streptomycin (0.5mg/ml) were added to the extenders. Semen was diluted to a final concentration of 0.8 × 10^9 spermatozoa/ml and then stored at 5°C. Quality of stored semen was assessed at 0, 8 and 24 h.

Semen evaluation

Motility

The percentages of total and progressive motile spermatozoa in each sample were determined using a computer-assisted sperm analysis system (ISAS, version 1.0.17, Proiser, Valencia, Spain) as described by Yániz *et al*., (2008). Semen samples were diluted with NaCl 0.9% to reach 20 × 10^6 sperm/ml and a minimum number of 200 spermatozoa from three to four different fields were assessed.

Viability

Sperm viability was evaluated with eosin-nigrosin staining (Evans and Maxwell, 1987). Smears were prepared by mixing a 2.5 µl of semen (diluted at 20 × 10^6 spermatozoa/ml) with 2.5 µl of the eosin-nigrosin on a warm slide. The viability was assessed by counting 200 cells under bright-field microscopy (400 x). Spermatozoa showing partially or completely stained (pink to red) head was considered non-viable or dead and only sperm showing strict exclusion of the stain were considered to be alive (Chauhan and Anand, 1990).

Morphology

Smears were prepared from 5 µl diluted sperm samples on slide previously cleaned with absolute methanol and stained with Diff-Quick® (Automatic Diagnostic Systems S.L. Barcelona, Spain). The slides were air-dried for 4 min, fixed for 1 min in Diff-Quik® fixative (Contains 0.002g/L of Fast Green in Methanol) prior to staining with Diff-Quik® solution 1 (Contains 1.22 g/L of EosinY in phosphate buffer at pH 6.6 and 0.1% (w/v) sodium azide as preservative) for 50 s and with Diff-Quik® solution 2 (Contains 1.1 g/l of Thiazine Dye in phosphate buffer at pH 6.6) for 50 s. Excess solutions were removed by placing the slides vertically on absorbent paper between each step. At least 200 spermatozoa were assessed with a UB203 microscope (1000x magnification).

Sperm plasma membrane integrity

Sperm plasma membrane integrity was determined using hypo-osmotic swelling (HOS) assay as described by Revell and Mrode (1994). A 100 mOsM solution was prepared with fructose (9 g) and sodium citrate (4.9 g) in a liter of distilled water. To assess sperm plasma membrane integrity, 50 µl of semen was mixed with 500 µl of HOS solution and incubated for 60 minutes at 37°C. After incubation, 300 µl of the mixture was placed on a warm slide and cover slipped. One hundred spermatozoa were evaluated using a phase contrast microscope (400x) UB203. Spermatozoa with coiled tails were considered to have good membrane integrity.

Lipid peroxidation measurements

Lipid peroxidation (LPO) was determined in diluted samples by measuring the amount of thiobarbituric acid reactive species (TBARS) formed, according to a modified procedure described by Maia (2006). One ml of the TBA reagent (trichloracetic acid 15%, w/v, hydrochloric acid 0.25 N, thiobarbituric acid 0.375% w/v in distilled water) and 1% (v/v) of BHT (Butylated hydroxytoluene) solution at 50 mM were added to the 250 µl sample diluted with (Tris–hydroxymethyl-aminomethane 1.8184 g, monohydrated citric acid 0.9901 g, double distilled water up to 50 ml, pH 7.4) freshly prepared to obtain a volume of 500 µl content 10^8 sperm/ml. The mixture was heated at 100°C for 15min and then allowed to cool in ice. The sample was centrifuged at 1000 × *g* for 10 min. The supernatant was collected and TBARS were quantified by a spectropho-

tometer at 532 nm against a blank prepared under similar conditions. The amount of TBARS was calculated using a molar extinction coefficient of $1.56\times10^{-5}M^{-1}cm^{-1}$ for thiobarbituric acid and expressed in nmol TBARS/10^8 sperm.

Statistical analysis

Results are expressed as the mean ± SEM. Data were analyzed statistically using JMP11.0 (SAS Institute Inc., Cory, NC, USA) program. The data of extended semen quality parameters were analyzed by a factorial design ANOVA. The statistical model included the fixed effect of the extender, ram group, and storage periods. When statistically significant differences were detected, the Tukey's post hoc and Student's t-test were used to compare the means and standard errors, considering the significance level of ($P < 0.05$).

RESULTS

Experiment results are reported in tables 1-4. All factors studied (extender, storage time, age group and their interactions) had statistical significant effect on PM ($P < 0.01$) and LPO ($P < 0.01$). However, TM was affected only by age group*storage time ($P < 0.01$). Viability was affected by storage time ($P < 0.05$) and extender*storage time ($P < 0.05$). The percentage of sperm abnormalities was significantly affected by storage time ($P < 0.001$) and age group*storage time ($P < 0.05$). Finally, HOST was affected by storage time ($P < 0.001$), extender*storage time ($P < 0.001$) and age group * storage time* extender ($P < 0.05$) (table 1).

There was no significant effect of age and storage time on TM at 0h and 8h storage ($P > 0.05$).

However, TM was significantly better in the M group at 24 h ($P < 0.05$).

Table 1: Significance based on ANOVA for the effects of age, extender, storage time and the interactions extender × age, extender × storage time, age ×storage time and age × extender × storage time on motility, membrane integrity, abnormality, viability and lipid peroxidation

	PM (%)	TM (%)	VIAB (%)	ABN (%)	HOST (%)	LPO
Storage time (h)	***	NS	*	***	***	***
Extender* Storage time (h)	***	NS	*	NS	***	***
Group* Storage time (h)	**	**	NS	*	NS	***
Extender*Group*Storage time	***	NS	NS	NS	*	**

NS: not significant.*$P< 0.05$. ** $P < 0.01$. *** $P < 0.001$.

TM: total motility; PM: progressive motility; VIAB: viability; HOST: Hyperosmotic swelling test; ABN: abnormality. LPO: lipid peroxidation.

Table 2: Sperm quality during storage in two ram age groups in Skim milk and Tris egg yolk at 5°C

Group	Extender	Time (h)	PM (%)	HOST (%)	LPO
Group M	Skim milk	0	74.16 ± 1.25^{aA}	77.28 ± 0.56^{aA}	0.87 ± 0.02^{cA}
	Skim milk	8	63.07 ± 1.39^{bA}	71.12 ± 0.51^{bA}	1.31 ± 0.03^{bB}
	Skim milk	24	63.50 ± 0.43^{bA}	68.16 ± 0.70^{cA}	2.28 ± 0.03^{aA}
	Tris egg yolk	0	65.71 ± 1.48^{aA}	75.83 ± 0.60^{aB}	0.79 ± 0.02^{cB}
	Tris egg yolk	8	47.64 ± 0.83^{bB}	71.00 ± 0.58^{bB}	1.49 ± 0.02^{bA}
	Tris egg yolk	24	44.07 ± 1.56^{bB}	66.16 ± 0.70^{cB}	2.11 ± 0.03^{aB}
Group A	Skim milk	0	63.66 ± 1.32^{aA}	73.33 ± 0.60^{aA}	1.08 ± 0.02^{cA}
	Skim milk	8	53.00 ± 1.69^{bA}	68.57 ± 0.72^{bA}	1.60 ± 0.02^{bB}
	Skim milk	24	46.33 ± 1.05^{cA}	65.5 ± 0.76^{cA}	2.82 ± 0.07^{aA}
	Tris egg yolk	0	53.21 ± 2.15^{aB}	72.5 ± 0.76^{aA}	0.93 ± 0.02^{cB}
	Tris egg yolk	8	41.25 ± 1.48^{bB}	65.5 ± 0.70^{bB}	1.91 ± 0.03^{bA}
	Tris egg yolk	24	28.58 ± 1.35^{cB}	57.16 ± 0.79^{cB}	2.52 ± 0.03^{aB}

PM: progressive motility; HOST: Hyperosmotic swelling test; ABN: abnormality. LPO: lipid peroxidation expressed in nmol of TBARS/10^8 sperm.

Values are expressed as mean ± SEM. Different superscripts (a, b, c) within the same column indicate a significant effect of storage time within each extender ($P<0.05$). Different superscripts (A, B, C) within the same row indicate a significant effect of extender within each time point ($P<0.05$).

Sperm abnormalities (ABN) were higher in the aged rams and increased in both age groups after 8 h of storage (P <0.05) (Table 1 and 4).

The PM decreased with storage time in both age groups (P <0.05) but was generally better in the M group (P <0.05). At 0h, there was no significant difference between the two extenders in terms of PM (P> 0.05). However, the decrease in PM was significantly more pronounced in TEY than in SM extender (P <0.05) (Table 1, 2 and 4).

Sperm viability was not affected by storage duration in the two extenders (P> 0.05). However, it was significantly higher in TEY (Table 3).

Lipid peroxidation (LPO) increased significant with storage time in both age groups and extenders (P<0.05). However, sperm from aged rams and sperm extended in SM was more affected than sperm from young mature rams and sperm extended in TEY extender (Tables 1, 2, and 4).

The HOST values decreased significantly with storage duration from 0h to 24h (P<0.05). There was a significant difference between the two extenders after 8 hours of storage. Membrane integrity was better in SM than TEY. The HOST values were significantly better for the M group compared to the A group in both extenders (P <0.05) (Table 2 and 4).

DISCUSSION

The present study is consistent with previous reports in that sperm quality parameters decrease significantly with storage duration (de Paz *et al*., 2010).

López-Sáez *et al*., (2000), reported that quality of spermatozoa decreases as the duration of storage increases independently of extenders, dilution rate, temperature or conditions of storage. The possible physiological reasons for this might be extracellular oxidative stress (Hong *et al*., 2010; de Lamirande *et al*., 1997; Maxwell and Salamon, 1993; Vishwanath and Shannon, 2000). However, liquid storage of ram spermatozoa at reduced temperature can inhibit metabolic activity which may contribute to improvement of survival rate (Anel *et al*., 2006). Our results clearly show a significant effect of age and type of extender on the quality of Boujaâd ram semen after storage. A previous study in our laboratory showed a superiority of TEY over SM for the preservation of Boujaâd ram semen in a liquid state at 5°C (Allai *et al*., 2015). In the present study semen stored in SM showed better motility and membrane integrity than semen stored in TEY. However, semen storage in TEY had less lipid peroxidation. This finding is in agreement with those of Tekin and Daşkin. (2016), who reported that motility of ram semen was better in SM than in TEY. Egg yolk seems to provide better viability and protection from oxidative stress (lower LPO) as suggested by Quan *et al*., (2016).

The difference between extenders may be explained by a difference in mechanisms of sperm protection during storage. SM casein micelles inhibit binding and prevent the adverse effects of BSP proteins on the spermatozoa membrane while maintaining the motility and viability of sperm during storage (Bergeron et *al*., 2007). In addition, the success of this diluent has been attributed to its protein fraction, which may act as a buffer against changes in pH and as a chelating agent against heavy metals (Salamon and Maxwell, 2000). Low-density lipoproteins (LDL) are the egg yolk constituents responsible for sperm protection (Amirat et *al*., 2004) against the adverse effects of BSP proteins during storage (Bergeron *et al*., 2004).

They associate with and stabilize the membrane (MacDonald and Foulkes, 1981) by forming a protective film on the sperm surface (Quinn *et al*., 1980) or by replacing sperm membrane phospholipids that are lost or damaged during

Table 3: Sperm viability (VIAB %) during storage at 5°C

Extender	Skim milk			Tris egg yolk		
Time (h)	0 h	8 h	24 h	0 h	8 h	24 h
VIAB (%)	91.12 ± 0.79^{aA}	88.4 ± 1.35^{abB}	86.25 ± 1.10^{bB}	92.33 ± 0.39^{aA}	92.16 ± 0.68^{aA}	91.91 ± 0.53^{aA}

VIAB: viability. Values are expressed as mean ± SEM. Different superscripts (a, b, c) indicate a significant effect of time within each extender (P<0.05). Different superscripts (A, B, C) indicate a significant effect of extender within each time point (P<0.05)

Table 4: Sperm quality during storage in the two ages group of ram at 5°C

Group	Time (h)	TM%	PM%	ABN%	LPO
Group A	0	88.73 ± 0.83^{aB}	57.34 ± 1.62^{aB}	8.46 ± 0.23^{bB}	1.02 ± 0.02^{cA}
	8	85.89 ± 1.39^{aB}	45.77 ± 1.44^{bB}	9.30 ± 0.44^{bB}	1.74 ± 0.04^{bA}
	24	86.61 ± 1.15^{aB}	34.5 ± 2.24^{cB}	13.75 ± 0.50^{aB}	2.67 ± 0.06^{aA}
Group M	0	90.78 ± 0.58^{bA}	68.53 ± 1.25^{aA}	6.77 ± 0.30^{bA}	0.83 ± 0.02^{cB}
	8	92.07 ± 0.56^{aA}	53.18 ± 1.39^{bA}	7.64 ± 0.37^{bA}	1.39 ± 0.03^{bB}
	24	90.55 ± 0.56^{bA}	49.9 ± 2.45^{bA}	10.16 ± 0.36^{aA}	2.19 ± 0.03^{aB}

TM: total motility; PM: progressive motility, ABN: abnormality, LPO: lipid peroxidation expressed in nmol of TBARS/10^8 sperm. Values are expressed as mean ± SEM. Different superscripts (a, b, c) within the same column indicate a significant effect of storage time within each extender (P<0.05). Different superscripts (A, B, C) within the same row indicate a significant effect of two age group within each time point (P<0.05).

the process (Graham and Foote, 1987). It is evident in the present study that sperm derived from the young mature ram maintains better quality and viability during liquid storage compared to sperm from older rams. This may be inherent to the initial quality of semen which is reportedly better in young mature rams than in older rams (Hassan, *et al*., 2009; Mahmood *et al*., 2014). Several factors may be involved in this effect of age on quality post storage including differences in seminal plasma composition and spermatogenic activity. Studies in humans have shown that the incidence of spermatozoa with severely damaged DNA increases significantly with age (Vagnini *et al*., 2007; Schmid *et al*., 2007). More studies on molecular aspects of DNA changes and apoptosis would be useful in determining the mechanism of the effect of age on sperm quality and its ability to withstand storage.

CONCLUSION

Our results confirm that the age of rams has a significant effect on initial quality of ejaculated and their ability to withstand cooling and preservation in the most common type of extenders. In addition, it was confirmed that SM and EY extenders provide protection to sperm during storage in different ways. EY seems to be protective against oxidative stress while SM seems to preserve motility. Artificial insemination trials are necessary to determine if these differences have an effect on fertilizing ability or not.

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