Effect of extender and storage temperature on sperm motility parameters of liquid ram semen

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Abstract

The objective of this work was to compare the effect of eight extenders (Skim milk, INRA96®, Tris Eggs Yolk, Triladyl®, Ovipro®, Andromed®, Optixcel® and Duragen®) on ram sperm motion parameters. Two experiments were carried out to determine the effect of extenders on ram sperm preservation at 15°C (Experiment 1), or at 5°C (Experiment 2). Semen was collected using an artificial vagina weekly for 10 weeks from two breeds (INRA180, n=4, on Monday and Boujaâd, n=4, on Friday). Aliquots were extended in each extender to a final concentration of 0.8×10^9 spermatozoa/ml and stored at 15°C or 5°C for 24 h. Motility and kinematic parameters assessment were performed using a CASA system. Sperm motility and kinematic parameters decreased significantly over time of storage in all extenders and storage temperature. The overall CASA parameters were affected by extenders in different manners depending on breed and storage temperature. Skim milk, Duragen®, and INRA96® provided the best CASA results.

Keywords: Semen, extender, motility, kinematic parameters, storage temperature, ram breed

Effet du dilueur et de la température du stockage sur les paramètres de motilité des spermatozoïdes du bélier conservés à l'état liquide

Résumé

L'objectif de ce travail était de comparer l'effet de huit dilueurs (lait écrémé, INRA96®, Tris jaune d'œuf, Triladyl®, Ovipro®, Andromed®, Optixcel® et Duragen®) sur les paramètres du mouvement du sperme du bélier. Deux expérimentations ont été réalisées pour déterminer l'effet des dilueurs sur la conservation du sperme de bélier à 15°C (expérimentation 1), ou à 5°C (expérimentation 2). Le sperme a été collecté à l'aide d'un vagin artificiel chaque semaine durant 10 semaines des deux races ovines (INRA180, n=4, lundi et Boujaâd, n=4, vendredi). Des aliquotes du sperme ont été diluées dans chaque dilueur à une concentration finale de 0,8 x 10^9 spermatozoïdes / ml et conservées à 15°C ou 5°C durant 24 heures. La motilité et les paramètres cinétiques ont été évalués à l'aide d'un système CASA. La motilité des spermatozoïdes et les paramètres cinétiques ont diminué significativement au cours du temps de stockage dans tous les dilueurs et les températures de stockage. Les dilueur ont affecté les paramètres générés par le système CASA de différentes manières en fonction de la race et de la température du stockage. Le lait écrémé, Duragen® et INRA96® ont fourni les meilleurs résultats de CASA.

Mots-clés: Sperme, dilueurs, motilité, paramètres cinétiques, température du stockage, race du bélier

INTRODUCTION

The necessity to fertilize large numbers of ewes with semen from outstanding rams, requires the transport of semen from the points of collection to the sites of insemination. This could be achieved by two major methods that reduce the metabolism of spermatozoa and thereby prolonged their fertile life: storage in liquid form or cryopreservation. Artificial insemination with frozen-thawed ram semen has not been extensively adopted in the world due to the complex anatomy of the ewe cervix (Rodriguez *et al*., 1988). Additionally, the use of frozen semen requires sophisticated equipment and advanced training (laparoscopy, special equipment for cervical catheterization) which precludes its use in a large scale program. Artificial program insemination using liquid stored semen is more appropriate for most field conditions and results in high pregnancy rates (Quan *et al*., 2016).

Ram semen can be stored for various lengths of time at a temperature of 0 to 5°C or 10 to 15°C (Salamon and Maxwell, 2000). The chemical composition of extenders is critical for successful liquid storage of ram spermatozoa in this range of temperature. Several extenders are available and differ in their basic composition (milk, egg yolk, soya lecithin. Liposome etc.) (Salamon and Maxwell, 2000). Some of these extenders were specifically formulated for ovine semen, while others were formulated for other species and adapted to ovine semen (Sexton, 1988; Fukui *et al*., 2008; Forouzanfar *et al*., 2010).

The motility of spermatozoa is one of the most commonly assessed parameters for evaluation of semen quality. Motility can be assessed objectively using computer-assisted sperm analyzer (CASA) which provide several parameters characterizing sperm motion in addition to the traditional total and progressive motility. Studies using these tech-

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niques showed a high positive correlation between sperm curvilinear velocity (Vcl), straight-line velocity (Vsl), and average-path velocity (Vap) and pregnancy rate (Donnelly *et al*., 1998). Other studies suggest that progressive motility is a potential predictive parameter for sperm fertilizing capacity (Li *et al*., 2016; Herrara *et al*., 2005).

The aim of the present study was to determine the effect of extenders (Skim Milk (SM), INRA96®, Tris Eggs Yolk (TEY), Triladyl®, Ovipro®, Andromed®, Optixcel® and Duragen®) on ram sperm motion parameters after 24 h of liquid storage at 5 and 15°C. The same experimental procedures were conducted in 2 breeds of rams (INRA180 and Boujaâd) during the same period of the year.

MATERIAL AND METHODS

Two experiments were designed to investigate the effect of extender on INRA180 and Boujaâd ram semen motion parameters after storage for 24 h at 15°C (Experiment 1) or at 5°C (Experiment 2). For each experiment, semen was collected weekly for 10 weeks (Experiment 1: from March 9th until May 18th, 2015) (Experiment 2: from July 1st until September 21st, 2015). Samples were extended in skim milk (SM) (Colas *et al*., 1968), INRA96® (IMV Technologies, France), Tris egg-yolk (TEY) (Paulenz *et al.*, 2002), Triladyl®, Ovipro®, Andromed® (Minitube, Germany), Optixcel® (IMV Technologies, France) and Duragen® (Magapor S.L.; Zaragoza, Spain) extenders and stored at 15°C or 5°C for 24 h.

Animals and semen collection and initial evaluation

Eight mature, healthy rams (age 2 to 4 years), 4 each of two breeds INRA180 and Boujaâd rams were used in the experiments. Animals were housed under semi-arid conditions at the National Institute of Agricultural Research center of Settat; Morocco (32◦ latitudes). Ejaculates were collected using an artificial vagina on a weekly basis (INRA180 ram on Mondays and Boujaâd rams on Fridays). Ejaculates were placed immediately in a water-bath at 37°C. Ejaculates were assessed for volume, concentration and motility. Initial sperm mass motility (x100) and individual motility (x200) were evaluated subjectively. Sperm concentration was assessed with a spectrophotometer previously calibrated by hemocytometry. Samples with a concentration of >2.10^{9} spermatozoa/ml; mass motility >3, and individual motility >70 % were used in the experiments.

Semen processing

Ejaculates that met or exceeded the minimum requirements were divided into equal aliquots and each aliquot was diluted one to one (semen: extender) with one of the eight extenders. All extenders were prepared according to manufacturer recommendations. Diluted semen samples were placed in a water bath at 37°C and concentration was adjusted to 0.8 10^{9} spermatozoa/ml. After an initial assessment, extended semen samples were stored at the desired temperature (15°C for the first experiment and 5°C for the second experiment).

Extended semen was assessed before cooling (0 h), at 8 h and 24 h of storage for motility, using a computer-assisted sperm motility analysis system (CASA; ISAS, version 1.0.17, Proiser, Valencia, Spain) as described by Yániz *et al*., (2008). Parameters recorded at each analysis time included: sperm total motility (MT), progressive motility (PM), curvilinear velocity (Vcl), straight-line velocity (Vsl), and average path velocity (Vap). Semen was diluted in phosphate buffered saline supplemented with bovine serum albumin (1 mg/ml) to achieve 2.10^{6} spermatozoa/ml prior to analysis.

Statistical analysis

The statistical analyses were performed using JMP SAS 11.0.0 (SAS Institute Inc., Cory, NC, USA) program. It is important to note that these experiments were not designed to compare breed performance and storage temperature simultaneously. Analysis of the extender effect was performed for each breed of rams separately. Semen motility and kinematic parameters were analyzed by a factorial design ANOVA. The statistical model included the fixed effect of the base extender, and storage periods (0, 8, and 24 h). When statistically significant differences were detected, the Tukey's post hoc was used to compare the means and standard errors, considering the significance level of $P < 0.05$. Data are expressed as the mean ± SEM.

RESULTS

Experiment 1: Effect of extenders on ram semen storage at 15°C

Effect of extenders on INRA180 ram semen storage at 15°C

Motility parameters obtained by CASA for INRA180 ram sperm stored at 15°C in different extenders are presented in Table 1 and 2. There was a significant negative effect of storage time on all motility parameters. Total motility and Vap decreased significantly with storage duration from 0 h onward. While, Vcl decreased significantly from 0 h to 8 h of storage (Table 1).

Table 1: Effect of storage time on TM (Total motility), Vcl (curvilinear velocity), and Vap (average-path velocity) of INRA180 ram sperm stored at 15°C

Storage time (hours)	TM (%)	Vcl (µm/s)	Vap (µm/s)
0	86.5±0.37^{a}	197.71±1.97^{a}	122.31±0.89^{a}
8	82.48±0.69^{b}	183.43±2.34^{b}	117.33±0.84^{b}
24	79.41±0.69^{c}	176.46±1.97^{b}	105.15±1.03^{c}

Different superscripts within columns indicate a significant effect of storage time within each parameter ($P < 0.05$).

As shown in Table 2, PM remained unchanged in SM throughout the storage time ($P > 0.05$). While for semen stored in Ovipro® and TEY, there was a marked decrease from 0 h to 8 h of storage ($P < 0.05$). Progressive motility decreased markedly from 8h of storage onward in Duragen® and INRA96® ($P < 0.05$).

A significant decrease in Vsl was observed between 0h to 8 h in SM and TEY. Whereas, in Duragen® and INRA96®, Vsl decreased from 8 h of storage onward ($P < 0.05$). Straight-line velocity declined significantly in Ovipro® samples from 0 h to 24 h.

Progressive motility and Straight-line velocity were better maintained in Duragen®, SM and INRA96® than in other extenders. Andromed® and Optixcel® recorded the most significant decline, particularly after 24 h of storage (Table 2).

Effect of extenders on Boujaâd ram semen storage at 15°C

Motility parameters obtained by CASA for Boujaâd ram sperm stored at 15°C in different extenders are presented in Table 3, 4 and 5. There was a negative effect ($P < 0.05$) of storage time on all motility parameters. There was no significant effect of storage time on the PM in SM extender. Semen stored in TEY showed a significant decrease in PM from 0 h to 8 h. Progressive motility, decreased from 8 h to 24 h in INRA96®, Optixcel® and Triladyl® ($P < 0.05$) and from 0 h to 24 h in Andromed®, Duragen® and Ovipro® ($P < 0.05$). Progressive motility was significantly advanced in SM and lower in TEY, Optixcel® and Andromed® particularly after 24 h of storage. The remaining extenders had an intermediary effect (Table 3). Sperm TM was maintained for 24 h of storage in Andromed®, INRA96®, SM, Optixcel®, Ovipro®, Triladyl® and TEY ($P > 0.05$). For semen extended in Duragen®, TM decreased significantly with storage duration (form 0 h to 24 h). Total motility was significantly lower in Duragen® compared to the other extenders particularly at 24 h of storage. Andromed®, INRA96®, SM, Optixcel®, Ovipro®, Triladyl®, and TEY had similar TM regardless of storage time ($P > 0.05$) (Table 3).

Straight-line velocity decreased by 8 h of storage in Ovipro®, TEY, and Triladyl® and from 8 to 24 h of storage for semen stored in Andromed®, Duragen®, INRA96®, SM and Optixcel® ($P < 0.05$). Straight-line velocity was higher in Duragen® and SM and lower in Andromed®. While the other extenders gave an intermediary Vsl result (Table 4).

Semen extended in Andromed®, Duragen®, INRA96®, and SM extenders showed a significant decrease in Vap between 8 and 24 h of storage. However, semen stored in

Table 2: Effect of storage time and extender on PM (Progressive motility) and Vsl (Straight-line velocity) in INRA180 ram sperm stored at 15°C

Extender	PM (%)			Vsl (µm/s)		
	0 h	8 h	24 h	0 h	8 h	24 h
Andromed®	60.16 ± 1.45^{aB}	47.75 ± 2.27^{bB}	23.83 ± 2.07^{cC}	92.14 ± 1.51^{aD}	77.39 ± 2.01^{bB}	54.67 ± 2.51^{cCD}
Duragen®	66.96 ± 1.05^{aA}	63.14 ± 1.19^{aA}	54.21 ± 2.02^{bA}	106.49 ± 2.46^{aB}	105.17 ± 2.36^{aA}	90.59 ± 2.59^{bA}
INRA96®	62.62 ± 1.63^{aAB}	61.48 ± 1.36^{aA}	53.4 ± 1.99^{bA}	104.78 ± 3.32^{aBC}	97.25 ± 1.89^{aA}	86.5 ± 2.77^{bA}
SM	65.57 ± 1.23^{aAB}	65.13 ± 1.22^{aA}	61.78 ± 2.09^{aA}	169.69 ± 2.92^{aA}	99.83 ± 2.24^{bA}	92.84 ± 2.72^{bA}
Optixcel®	58.94 ± 1.58^{aB}	41.69 ± 2.94^{bB}	22.41 ± 1.78^{cC}	90.27 ± 2.36^{aD}	73.94 ± 3.18^{bB}	50.89 ± 1.66^{cD}
Ovipro®	58.96 ± 1.55^{aB}	40.67 ± 2.21^{bB}	35.73 ± 1.89^{bB}	93.05 ± 2.16^{aCD}	76.27 ± 2.71^{bB}	62.23 ± 2.61^{cBC}
TEY	48.1 ± 2.24^{aC}	37.41 ± 0.62^{bB}	36.96 ± 3.46^{bB}	85.78 ± 2.85^{aD}	74.63 ± 3.45^{bB}	68.92 ± 3.09^{bB}
Triladyl®	59.21 ± 1.42^{aB}	45.52 ± 2.22^{bB}	35.06 ± 2.11^{cB}	91.96 ± 1.73^{aD}	80.12 ± 2.22^{bB}	60.88 ± 2.69^{cBCD}

a, b, c. Different superscripts within rows indicate an effect of storage duration within each extender ($P < 0.05$).
A, B, C. Different superscripts columns indicate an effect of extender for each storage duration ($P< 0.05$).

Table 3: Effect of storage time and extender on PM (Progressive motility) and TM (Total motility) in Boujaâd ram sperm stored at 15°C

Extender	PM (%)			TM (%)		
	0 h	8 h	24 h	0 h	8 h	24 h
Andromed®	66.75 ± 1.38^{aA}	44.45 ± 2.22^{bC}	30.14 ± 2.49^{cD}	89.11 ± 1.05^{aA}	85.65 ± 1.72^{abA}	81.6 ± 1.83^{bA}
Duragen®	66.18 ± 1.29^{aA}	55.3 ± 1.84^{bAB}	45.57 ± 1.99^{cB}	84.48 ± 1.19^{aB}	74.65 ± 1.69^{bB}	65.06 ± 1.93^{cB}
INRA96®	$63.56\pm1,87^{aAB}$	56.7 ± 1.91^{aAB}	46.09 ± 2.69^{bB}	87.12 ± 1.56^{aAB}	82.53 ± 2.08^{abA}	79.26 ± 2.84^{bA}
SM	66.71 ± 1.29^{aA}	62.38 ± 1.34^{abA}	57.96 ± 2.35^{bA}	84.25 ± 1.23^{aB}	81.98 ± 1.13^{aA}	75.96 ± 2.21^{bA}
Optixcel®	57.26 ± 1.82^{aB}	54.43 ± 1.96^{aAB}	33.74 ± 2.64^{bCD}	87.11 ± 1.02^{aAB}	84.62 ± 1.46^{abA}	78.77 ± 2.51^{bA}
Ovipro®	61.28 ± 1.39^{aAB}	51.69 ± 2.19^{bBC}	40.89 ± 2.01^{cBC}	88.31 ± 0.89^{aAB}	85.11 ± 1.52^{abA}	81.94 ± 1.48^{bA}
TEY	59.29 ± 2.34^{aB}	33.25 ± 2.48^{bD}	33.11 ± 1.69^{bCD}	87.75 ± 1.11^{aAB}	86.39 ± 1.13^{aA}	78.21 ± 1.64^{bA}
Triladyl®	61.33 ± 1.33^{aAB}	57.7 ± 1.72^{aAB}	39.21 ± 1.59^{bBC}	86.5 ± 1.15^{aAB}	85.35 ± 1.36^{aA}	82.83 ± 1.50^{aA}

a, b, c. Different superscripts within rows indicate an effect of storage duration within each extender ($P < 0.05$).
A, B, C. Different superscripts within columns indicate an effect of extender for each storage duration ($P < 0.05$).

Optixcel®, Ovipro®, TEY, and Triladyl® showed a steady decrease at all storage times (P < 0.05) (Table 4).

The curvilinear velocity was unaffected for up to 24 h in Ovipro® and Triladyl® (P >0.05) nonetheless decreased significantly between 8 h and 24 h of storage in Andromed®, Duragen®, INRA96®, SM and Optixcel® (P < 0.05). Semen stored in Ovipro® and Triladyl® recorded the highest sperm curvilinear velocity at 24 h. While the lowest Vcl results were obtained in SM and Duragen®. The other extenders had intermediary effect on sperm Vsl (Table 5).

Experiment 2: Effect of extenders on ram semen storage at 5°C

Effect of extenders on INRA180 ram semen storage at 5°C

Motility parameters obtained by CASA for INRA180 ram sperm stored at 5°C in different extenders are presented in Table 6, 7, and 8.

There was a significant negative effect (P<0.05) of storage time on all motility parameters. Progressive motility was significantly decreased from 0 h to 24 h in Andromed®, Optixcel® and Triladyl®). However, it decreased from 0 h to 8 h in Duragen® and INRA96® extenders (P < 0.05). This parameter decreased from 8 h to 24 h in SM, Ovipro® and TEY extenders (P < 0.05). Skim milk, INRA96® and Duragen® recorded the highest PM. Optixcel® showed the lowest PM, especially at 24h of liquid storage (Table 6).

Total motility was unchanged in SM, TEY, Optixcel®, and Ovipro® regardless of storage time (P > 0.05). However, this parameter was significantly decreased from 0h to 8 h in Andromed®, Duragen® and INRA96®, and from 8 h to 24 h in TEY, Ovipro®, and Triladyl® (P < 0.05). Total motility was slightly decreased in Duragen® compared to the other extenders (Table 6).

A decreased in Vcl was observed over time of storage increase in Duragen® and TEY (P < 0.05). This parameter decreased in SM extender between 0 h to 8 h of storage (P < 0.05). Sperm Vcl was not affected by storage duration in Andromed®, INRA96®, Optixcel®, Ovipro® and Triladyl® (P > 0.05). Sperm Vcl was better preserved in Andromed® and Ovipro® compared to the remaining extenders (Table 7).

Sperm Vsl decreased with increasing time of storage in Andromed®, Duragen®, Optixcel®, Ovipro®, TEY and

Table 4: Effect of storage time and extender on VSL (Straight-line velocity) and VAP (Average path velocity) in Boujaâd ram sperm stored at 15°C.

Extender	Vsl(µm/s)			Vap(µm/s)		
	0 h	8 h	24 h	0 h	8 h	24 h
Andromed®	96.69±2.42^{aBC}	94.15±15.76^{aAB}	55.78±2.79^{bD}	120.57±2.71^{aCD}	118.08±3.19^{aABC}	97.86±3.20^{bB}
Duragen®	111.08±1.80^{aA}	103.63±3.23aA	81.57±3.07^{bA}	135.23±2.14^{aA}	129.13±3.43^{aA}	99.67±3.11^{bAB}
INRA96®	109.09±3.52^{aA}	100.07±4.01^{aAB}	67.26±4^{bBCD}	131.94±5.56^{aABC}	128.43±3.87^{aAB}	94.74±4.77^{bB}
SM	96.65±2.16^{aBC}	93.52±2.75^{aAB}	82.61±2.92^{bA}	117.51±2.56^{aD}	115.86±2.57^{aBC}	99±3.19^{bAB}
Optixcel®	91.12±2.69^{aC}	82.31±2.79^{aAB}	61.98±3.23^{bCD}	123.41±2.42^{aBCD}	113.13±2.74^{bC}	94±2,94^{cB}
Ovipro®	102.5±1.85^{aAB}	86.5±2.56^{bAB}	75.28±2.24^{cAB}	134.78±2.25^{aAB}	121.46±3.07^{bABC}	110.3±2.28^{cA}
TEY	102.59±1.92^{aAB}	72.39±1.76^{bB}	58.29±2.29^{cCD}	137.51±1.68^{aA}	123.5±1.73^{bABC}	95.11±2.03^{cB}
Triladyl®	104.29±2.38^{aAB}	92.9±2.36^{bAB}	67.52±2.15^{cBC}	132.66±2.71^{aAB}	120.6±2.25^{bABC}	104.3±2.74^{cAB}

a, b, c. Different superscripts within rows indicate an effect of storage duration within each extender (P < 0.05).

A, B, C. Different superscripts within columns indicate an effect of extender for each storage duration (P < 0.05).

Table 5: Effect of storage time and extender on Vcl (Curvilinear velocity) in Boujaâd ram sperm stored at 15°C.

Extender	Vcl (µm/s)		
	0 h	8 h	24 h
Andromed®	221.75±5.79^{aB}	202.04±6.61^{bB}	191.34±3.73^{bB}
Duragen®	183.94±2.86^{aDE}	179.6±4.28^{aCD}	140.63±3.81^{bC}
INRA96®	203.13±6.44^{aBCD}	200.94±5.63^{aABC}	176.67±7.59^{bB}
SM	169.61±3.46^{aE}	162.22±3.76^{aD}	134.03±3.82^{bC}
Optixcel®	200.73±4.50^{aCD}	200.79±5.26^{aBC}	181.69±6.19^{bB}
Ovipro®	218.89±3.46^{aB}	218.24±3.19^{aAB}	218.73±4.32^{aA}
TEY	250.56±3.92^{aA}	224.29±4.84^{bA}	194.29±4.86^{cB}
Triladyl®	203.60±3.45^{aBC}	199.79±4.99^{aBC}	199.02±4.63^{aAB}

a, b, c. Different superscripts within rows indicate an effect of storage duration within each extender (P < 0.05).

A, B, C. Different superscripts within columns indicate an effect of extender for each storage duration (P < 0.05).

Triladyl® ($P < 0.05$). The decrease was marked from 0 h to 8 h in SM ($P < 0.05$) and from 8 h to 24 h in INRA96® ($P < 0.05$). Duragen®, INRA96® and SM preserved sperm Vsl better compared to the other extenders. Optixcel® recorded the lowest Vsl value. While, the remaining extenders were intermediary (Table 8).

Sperm Vap decreased significantly from 0 h to 24 h in Duragen®, Ovipro®, and TEY. The decrease in sperm Vap was recorded from 0h to 8 h in Andromed® and INRA96® ($P < 0.05$) and from 8 h to 24 h in SM, Optixcel® and Triladyl® ($P < 0.05$). Triladyl® and TEY presented the lowest Vap compared to the other extenders especially at 24 h of storage (Table 8).

Table 6: Effect of storage time and extender on PM (Progressive motility) and TM (Total motility) in INRA180 ram sperm stored at 5°C

Extender	PM (%)			TM (%)		
	0 h	8 h	24 h	0 h	8 h	24 h
Andromed®	60.9±1.27^{aA}	35.76±3.23^{bCD}	22.13±2.05^{cC}	92.14±2.36^{aBA}	79.22±2.91^{bAB}	74.46±2.91^{bA}
Duragen®	65.04±2.02^{aA}	47.14±1.85^{bB}	40.13±2.06^{bB}	81.82±1.58^{aB}	63.77±2.58^{bC}	59.21±2.92^{bB}
INRA96®	62.33±2.70^{aA}	48.58±2.36^{bB}	44.6±1.93^{bAB}	79.85±2.04^{aB}	70.73±2.37^{bBC}	68.31±2.09^{bAB}
SM	64.55±1.71^{aA}	59.42±1.94^{aA}	51.52±1.83^{bA}	82.13±1.27^{aB}	76.8±1.71^{abAB}	72.74±1.94^{bA}
Optixcel®	47.93±2.22^{aB}	27.04±3.28^{bD}	9.02±1.36^{cD}	80.2±1.30^{aB}	76.87±2.62^{abAB}	70.36±2.50^{bA}
Ovipro®	44.83±1.89^{aB}	44.25±2.18^{aBC}	17.91±1.98^{bC}	85.03±1.56^{aB}	82.11±1,71^{abA}	76.79±2.07^{bA}
TEY	35.68±2.59^{aC}	30.21±1.061^{aD}	18.21±1.51^{bC}	84.18±1.37^{aB}	80.52±1.79^{abA}	76.06±1.70^{bA}
Triladyl®	57.52±1.9^{aA}	49.88±2.05^{bAB}	21.19±2.21^{cC}	81.63±1.17^{aB}	80.69±1.40^{aAB}	69.3±2.48^{bAB}

a, b, c. Different superscripts within rows indicate an effect of storage duration within each extender ($P < 0.05$).

A, B, C. Different superscripts within columns indicate an effect of extender for each storage duration ($P < 0.05$).

Table 7: Effect of storage time and extender on Vcl (Curvilinear velocity) on INRA180 ram sperm stored at 5°C

Extender	Vcl (µm/s)		
	0 h	8 h	24 h
Andromed®	230.02±5.69^{aAB}	222.98±7.47^{aAB}	222.33±3.64^{aA}
Duragen®	200.31±2.86^{aCD}	181.03±4.77^{bDE}	157.15±4.41^{cD}
INRA96®	182.63±4.71^{aD}	178.57±5.18^{aDE}	176.14±5.26^{aCD}
SM	191.57±4.15^{aCD}	164.96±3.54^{bE}	158.98±5.47^{bD}
Optixcel®	216.67±6.20^{aBC}	197.88±5.53^{aBCD}	196.91±5.69^{bBC}
Ovipro®	230.93±6.59^{aAB}	220±6.73^{abABC}	206.22±5.12^{bAB}
TEY	252.25±7.20^{aA}	227.21±6.67^{bA}	196.09±4.81^{cBC}
Triladyl®	212.65±4.79^{aBC}	198.06±6.07^{aCD}	194.24±5.53^{aBC}

a, b, c. Different superscripts within rows indicate an effect of storage duration within each extender ($P < 0.05$).

A, B, C. Different superscripts within rows indicate an effect of extender for each storage duration ($P < 0.05$).

Table 8: Effect of storage time and extender on Vsl (Straight-line velocity) and Vap (Average path velocity) in INRA180 ram sperm stored at 5°C

Extender	Vsl (µm/s)			Vap (µm/s)		
	0 h	8 h	24 h	0 h	8 h	24 h
Andromed®	110±2.04^{aAB}	68.49±3.44^{bC}	55.63±2.80^{cB}	145.7±3.15^{aA}	111.59±3.29^{bD}	104.87±2.86^{bA}
Duragen®	126.65±3.54^{aA}	113.08±3.99^{bA}	87.51±2.71^{cA}	152.75±3.57^{aA}	136.56±4.06^{bA}	107.74±3.21^{cA}
INRA96®	115.72±4.62^{aA}	92.08±3.62^{bB}	81.43±1.47^{bA}	137.44±4.79^{aA}	117.55±3.51^{bBCD}	105.63±2.29^{bAB}
SM	115.6±3.49^{aA}	108.96±3.16^{aA}	86.72±1.68^{bA}	138.55±3.64^{aA}	129.13±3.13^{aAB}	110.24±1.62^{bA}
Optixcel®	82.27±2.52^{aC}	62.17±3.77^{bC}	36.09±1.74^{cD}	116.44±2.83^{aB}	113.29±2.65^{aCD}	101.24±1.67^{bABC}
Ovipro®	94.9±2.47^{aB}	83.03±3.13^{bB}	48.43±2.68^{cBC}	141.32±3.61^{aA}	122.75±3.33^{bABCD}	101.69±2.12^{cABC}
TEY	83.02±2.84^{aC}	63.52±2^{bC}	44.42±1.44^{cCD}	138.52±2.52^{aA}	115.79±2.98^{bCD}	92.92±1.84^{cC}
Triladyl®	109.52±4.36^{Ba}	91.42±3.04^{bB}	51.96±2.63^{cBC}	138.96±4.82^{aA}	126.25±2.69^{aABC}	94.28±2.62^{bBC}

a, b, c. Different superscripts within rows indicate an effect of storage duration within each extender ($P < 0.05$).

A, B, C. Different superscripts within columns indicate an effect of extender for each storage duration ($P < 0.05$).

Effect of extenders on Boujaâd ram semen storage at 5°C

The CASA results for Boujaâd sperm stored at 5°C in different extenders are presented in Table 9, 10 and 11. Regardless of extender type, Vsl and Vap were decreased significantly as the storage duration increase (Table 9).

Table 9: Effect of storage time on semen Vsl (Straight-line velocity) and Vap (Average path velocity) in Boujaâd ram sperm stored at 5°C

Time (hours)	Vsl(µm/s)	Vap(µm/s)
0	97.95±0.98^{a}	125.69±1.04^{a}
8	80.94±2.95^{b}	107.02±1.09^{b}
24	60.79±1.19^{c}	96.53±1.98^{c}

Different superscripts within columns indicate a significant effect of storage time within each parameter ($P < 0.05$).

Progressive motility decreased from 0 h to 24 h of storage ($P < 0.05$) in samples extended in Andromed®, INRA96®, Optixcel®, Ovipro®,and Triladyl® d. A decrease of PM was recorded only from 0 h to 8 h in SM, TEY and Duragen® ($P < 0.05$). Duragen® and SM registered the highest PM, particularly at 24 h of storage (Table 10).

Ovipro® did not affect the TM during storage ($P > 0.05$). Sperm TM decreased in Optixcel® from 0 h to 24 h ($P < 0.05$) and from 0h to 8h in SM, TEY, Triladyl®, Duragen® and INRA96® ($P < 0.05$). Total motility was higher in SM and Ovipro® compared to the other extender (Table 10).

Sperm Vcl was not affected by storage time ($P > 0.05$) in Andromed® and Triladyl®. This parameter decreased during storage between 0 and 24 hours in Duragen® ($P < 0.05$). A decrease in sperm Vcl was observed from 0 h to 8 h in TEY, Optixcel®, and INRA96® and from 8 h to 24 h in Ovipro® and SM ($P < 0.05$). Overall SM, Duragen®, and Optixcel® registered the most significant decline, particularly after 24 hours of storage (Table 11).

DISCUSSION

CASA provides an objective evaluation of sperm motility. This approach was used in the present study to evaluate the effect of several extenders (SM, TEY, Duragen®, Andromed®, Triladyl®, Optixcel®, INRA96®, and Ovipro®) on total motility, progressive motility, Vcl, Vsl, and Vap. Overall CASA parameters were significantly affected by the type of extender used in both breeds of ram (INRA180 and Boujaâd) regardless of the temperature of storage (5 or 15°C).

Table 11: Effect of storage time and extender on VCL (Curvilinear velocity) in Boujaâd ram sperm stored at 5°C

	Vcl (µm/s)		
Extender	**0 h**	**8 h**	**24 h**
Andromed®	209.42±4.85^{aA}	207.26±5.47^{aA}	199.87±6.03^{aA}
Duragen®	188.30±3.23^{aB}	175.07±4.08^{bB}	158.08±3.04^{cC}
INRA96®	216.74±3.32^{aA}	185.75±5.60^{bAB}	177.35±4.19^{bAB}
SM	200.79±3.59^{aA}	190.63±4.76^{aA}	141.07±4.04^{bD}
Optixcel®	211.87±6.27^{aA}	176.29±4.73^{bB}	171.56±9.53^{bC}
Ovipro®	234.47±6.44^{aA}	222.90±5.04^{abA}	203.14±5.99^{bA}
TEY	230.97±7.42^{aA}	199.67±6.23^{bAB}	199.71±9.11^{bA}
Triladyl®	193.22±5.99^{aAB}	189.2±5.58^{aA}	181.49±3.06^{aA}

a, b, c. Different superscripts within rows indicate an effect of storage duration within each extender ($P < 0.05$).

A, B, C. Different superscripts within columns indicate an effect of extender for each storage duration ($P < 0.05$).

Table 10: Effect of storage time and extender on PM (Progressive motility) and TM (Total motility) in Boujaâd ram sperm stored at 5°C

	PM (%)			TM (%)		
Extender	**0 h**	**8 h**	**24 h**	**0 h**	**8 h**	**24 h**
Andromed®	60.23±1.29^{aAB}	32.65±2.05^{bB}	14.11±1.32^{cD}	85.47±0.90^{aA}	74.49±1.99^{bABC}	71.18±2.09^{bBC}
Duragen®	64.45±1.43^{aAB}	46.67±3.03^{bA}	45.02±2.31^{bA}	82.39±1.34^{aA}	63.13±3.16^{bD}	62.57±2.46^{bCD}
INRA96®	58.37±1.48^{aBC}	43.96±2.40^{bA}	31.75±2.18^{cB}	82.48±1.21^{aA}	65.86±2.71^{bCD}	65.29±2.56^{bCD}
SM	65.87±1.30^{aA}	52.48±2.06^{bA}	51,98±2,43^{bA}	85.4±1.18^{aA}	78.55±2.10^{bAB}	76.18±2.37^{bAB}
Optixcel®	45.58±2.23^{aD}	25.97±2.85^{bB}	12.66±1.52^{cD}	80.77±1.44^{aA}	70.32±2.95^{bBCD}	60.93±2.61^{cD}
Ovipro®	53.22±1.58^{aC}	44.71±1.98^{bA}	28.51±1.93^{cBC}	83.49±1.55^{aA}	81.37±1,56^{aA}	80.4±1.43^{aA}
TEY	44.4±2.58^{aD}	25.18±1.82^{bB}	19.31±1.15^{bCD}	79.68±1.92^{aA}	66.77±2.11^{bCD}	59.88±2.71^{bCD}
Triladyl®	61.68±1.55^{aAB}	44.55±1.56^{bA}	30.37±2.45^{cB}	84.05±1.88^{aA}	78.26±1.98^{aAB}	69.39±1.84^{bBCD}

a, b, c. Different superscripts within rows indicate an effect of storage duration within each extender ($P < 0.05$).

A, B, C. Different superscripts within columns indicate an effect of extender for each storage duration ($P < 0.05$).

As expected, sperm motility parameters decrease over time of storage in the present study as reported previously (de Paz *et al.*, 2010). Liquid storage of ram semen has been extensively reviewed. López-Sáez *et al.,* (2000) reported that independently of diluents, dilution rate, temperature or conditions of storage, the quality of spermatozoa decreased as the duration of storage increased. The main change that occurs during storage is a reduction in motility. The possible physiological reason for this decline in motility may be extracellular oxidative stress (Hong *et al*., 2010; de Lamirande *et al.,* 1997; Maxwell and Salamon, 1993; Vishwanath and Shannon, 2000).

Our results suggest that various extenders preserve motility parameters during the 24h of storage through different mechanisms. It is interesting to note that extenders did not perform in exactly the same manner in the two breeds studied. At 15°C, SM, Duragen®, and INRA96® gave the best results after 24h for stored INRA180 ram semen. However, for Boujaâd ram semen, it seems that Ovipro®, Triladyl®, Duragen®, and SM were the best extenders for maintaining the motility parameters. For semen stored at 5°C, SM, INRA96®, Duragen®, Andromed®, and Ovipro® preserved motility parameters of INRA180 ram semen until 24 h. While for Boujaâd ram semen, results were better in SM, TEY, Duragen®, Ovipro®, INRA96®, Triladyl. Similarly, Curry *et al*., (1994) and Salamon and Maxwell (2000), acknowledged that the response to the conservation treatment, can vary from one breed to another and amongst males of the same breed. This could be due to differences in the biochemical composition of the ejaculate (Cabrera *et al*., 2005).

The sperm protective mechanism provided by extenders depends on their chemical compositions. For SM, the most protective constituents are casein micelles (the major proteins of milk). It has been shown that casein micelles isolated from milk can protect ram sperm (Choong and Wales, 1962), by preventing the binding of the major proteins of ram seminal plasma (RSP proteins) to sperm. Thus, preventing ram seminal plasma (RSP) protein-mediated stimulation of lipid loss from the membrane, during storage (Yue *et al*., 2009). Casein may prevent this interaction and damage of spermatozoa membrane. Phosphocaseinate-based extender (INRA96®) has also been shown to preserve the fertility of liquid ram semen stored between 5 to 15°C (Muro *et al*., 2006).

Egg yolk (EY) is generally added at a concentration of 20% (v/v) to the extender (Triladyl®, TEY, and Ovipro®). There is evidence that low-density lipoproteins (LDL) in the EY are responsible for sperm protection (Pace and Graham, 1974; Watson, 1976; Foulkes, 1977; Moussa *et al*., 2002; Amirat *et al*., 2004). However, the mechanism by which this protection is provided to sperm remains elusive. It is speculated that LDL is incorporated into the sperm membrane and provide protection by stabilizing it. However, there is contradictory evidence concerning the stability of the association of LDL to the sperm membrane (Watson, 1975; Foulkes, 1977; MacDonald and Foulkes, 1981). Another hypothesis suggests that phospholipids present in LDL protect sperm 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 the cryopreservation process (Foulkes *et al*., 1980; Graham and Foote, 1987). In bulls, it was reported that the interaction between a family of lipid binding proteins (BSP proteins) present in seminal plasma and LDL helps in the preservation of sperm integrity (Manjunath *et al*., 2002; Bergeron *et al*., 2004).

Recently, soybean lecithin has been used as an alternative to theeggyolk and used in commercial extenders for bull semen such as Andromed® (de Paz *et al*., 2010). This extender has been found useful for the preservation of the structural and functional integrity of ram spermatozoa (O'Hara *et al*., 2010) as well as it fertility following intracervical and intrauterine AI (Gil *et al*., 2003). On the other hand, O'Hara *et al.,* (2010), observed that storage of ram spermatozoa for 72 h at 5°C in Andromed® induced a marked decrease in the rate of in vitro blastocyst formation. Hiwasa *et al*., (2009), also detected a reduction in pregnancy rate of ewes inseminated intracervically with semen stored at 4°C in Andromed®. In the present study,milk, and egg yolk-based extenders achieved better results thanAndromed®.This is in agreement with Kulaksiz *et al*., (2012),who reported a better preservation of ram sperm motility in milk–egg extender than insoybean lecithin based extender for up to 48 h at 4°C. The decrease in motility in Andromed® is likely due to the high viscosity of soybean lecithin as reported by van Wagtendonk-de Leeuw *et al*., (2000).

Another approach to replace LDLs from EY and lecithin is the use of defined lipids such as liposomes (Gebauer *et al.*, 1970). The protective properties of liposomes are attributed to lipid and cholesterol transfer between liposomal and cellular membranes (Stewart *et al*., 2016). Optixcel® is a liposome-based extender designed for the preservation of bovine semen. Our results did not show any advantage of Optixcel® over the other extenders tested in the preservation of motility of ram sperm after storage at alow temperature in a liquid state.

Duragen® is an extra-long-term extender for boar sperm storage (Pinart *et al*., 2015). The exact composition of this commercial extender is proprietary, but must likely contain an energy source, protecting substrates against thermal shock, buffer salts, basic salts and antibiotics (Gadea *et al*., 2004). Except for a report from our laboratory (Amiri *et al*., 2016), this is the first study that tested Duragen® for liquid storage of ram semen. Our results with this extender are very promising and could be a good alternative to TEY and SM.

Semen quality may be affected by breed (Kasimanickam *et al.,* 2007), ambient temperature, semen collection frequency and age (Vilakazi, 2006). These factors may affect spermatogenesis or accessory glands function. Also, the storage temperature can affect the preservation of sperm depending on diluent used during the process (Paulenz *et al*., 2002). However, it is difficult to verify these effect from the present study because our experimental protocol was not designed to compare breeds (Boujaâd and INRA180) and storage temperatures (5 and 15°C) simultaneously (under the same experimental conditions).

CONCLUSION

From a practical point of view, our results may have a significant impact on the ovine industry, by giving more choices of extenders for the preservation of ram semen in the liquid state. Skim milk, Duragen®, and INRA96® gave the best motility results for INRA180 ram semen stored at 15°C. However, for Boujaâd ram semen, Ovipro®, Triladyl®, Duragen®, and SM were the best extenders for maintaining motility parameters. For storage at 5°C, SM, INRA96®, Duragen®, Andromed®, and Ovipro® are recommended for INRA180 ram semen. While for Boujaâd ram semen, results were better in SM, TEY, Duragen®, Ovipro®, INRA96®, Triladyl®. Nevertheless, CASA system parameters (motility and kinematic values) alone are not enough to evaluate the fertilization capacity of stored sperm. Other parameters such as viability, morphology, oviduct adhesion test, hypoosmotic swelling test, capacitation, DNA fragmentation and zona pellucida binding test should be investigated under the same condition. Ultimately, fertility trials will be necessary to confirm these results.

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