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Effect of ejaculation frequency on ram semen characteristics, seminal plasma composition and chilled sperm quality

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ABSTRACT

The aim of this study was to see how chilled sperm quality is affected by ejaculation frequency and its correlation to seminal plasma composition in INRA 180 rams. Five rams were collected at high (HFE) and low frequencies (LFE). For the high frequency, the rams were collected three times on the same day every three days for 18 days. In the low frequency collection, three consecutive ejaculates were collected once a week for four weeks. Ejaculates were collected at 20-minute intervals in either HFE or LFE. Semen characteristics, concentration of total protein, lipid, cholesterol and fructose in seminal plasma were assessed. Semen samples were extended in skim milk-based extender at 15 °C, then evaluated at different storage times (0, 8, and 24 h). Fresh sperm quality parameters, seminal plasma composition, and stored sperm quality were shown to be higher in LFE than in HFE and in the first and second ejaculates than in the third one. After 24 h of storage, sperm quality was correlated to seminal plasma components. In conclusion, the frequency of ejaculation has an effect on the fresh and stored semen quality as well as on seminal plasma composition in INRA 180 rams.

Keywords: Ram semen, seminal plasma, ejaculation rhythm, liquid storage

INTRODUCTION

The most important goal while collecting and handling semen is the efficiency of producing high-quality genetic material. During the artificial insemination process, the number of doses produced from one ejaculate and the number of adequate quality ejaculates are the two main parameters required from a ram (Faigl et al., 2012). Superior rams are often employed for mating or as sperm donors for genetic enhancement. Thus, a sufficient quantity of sperm with a high survival rate reaching the site of ovum fertilization ensures effective fertilization (Salamon and Maxwell, 1995). Nonetheless, sperm production is a critical issue, restricting long-term usage of rams across several ewes (Anel et al., 2005). Previous studies have shown that sperm quality parameters in rams decline drastically after successive ejaculates (Kaya et al., 2002). The INRA 180 sheep is a crossbred created by crossing D'Man and Timahdit. This breed has been demonstrated to inherit traits like prolificacy, sexual precocity, and non-seasonality (Benmoula et al., 2017). Nonetheless, the INRA 180 breed is regarded as one of the most endangered sheep breeds in Morocco, with just 500 extant heads, mostly protected through an *in-situ* program at the INRA-El Koudia station in Rabat and on several private farms. Consequently, intensifying semen

collection for an artificial insemination program provides a suitable technique for the *in-situ* conservation of this breed. However, there is no documentation on the effect of ejaculation frequency on the quality of sperm from INRA 180 rams. Furthermore, studies with different sheep breeds concentrating on the association between sperm quality and the frequency of successive ejaculations are contradictory. According to Kaya et al. (2002), with increased ejaculation frequencies, spermatozoa motility in the German Mutton Merino and Native Akkaraman rams crosse breed was lowered. In dissimilarity, in Gulf Coast Native rams, the second ejaculate had greater motility than the first one, collected at a 10-minute interval (Nel-Themaat et al., 2006). There was a negative correlation between sperm volume and sperm concentration, as well as enhanced motility in the third ejaculate following three consecutive 30-minute collections from Ile de France rams (Kistanova, et al., 2007). Besides, seminal plasma composition can be affected by collection frequency (Kaya et al., 2002) which can affect, in turn the preserved sperm quality (Ramírez-Vasquez et al., 2019). This makes specialists wondering how to employ rams as semen donors while preserving the greatest semen quality for a large number of sheep inseminations immediately after collection or after storage and transportation.

The goal of this study is to see how chilled sperm quality is affected by ejaculation frequency (HFE and LFE) and its correlation to seminal plasma composition in INRA 180 rams after 24 hours of storage.

MATERIALS AND METHODS

Animals and management

The animals are being studied in compliance with the Animal Use and Care standards of the University of Hassan 1st, Settat, Morocco.

Animals were maintained in semi-arid conditions at Morocco's National Institute of Agricultural Research in Settat (32° latitude). Five adult INRA 180 rams (2–3 years old) were kept under standard and comparable food, housing, and lighting settings. Lighting varied with the day length. The temperature ranged between 28 °C during the day and 15 °C at night. The health of the animals' external genitalia was clinically assessed. All animals were housed indoors in a covered shelter (500 m^2) and were free to roam. Rams were given a combination of straw–hay, barley, and sunflower. The daily amounts offered per head were 1000 g of hay, 800 g of barley and 300 g of sunflower.

Semen collection and initial evaluation

Five INRA180 rams were collected once every 3 days for 18 days at high frequency (HFE; N = 90), based on three ejaculates on the same day (30 first ejaculates, 30 second ejaculates and 30 third ejaculates). The ejaculates were collected around 20 minutes intervals. Concerning the low frequency collection (LFE; N = 60), it was performed after two months of rest (enough time for spermatogenesis), for 4 weeks. It was based on three ejaculates collected once every week (20 first ejaculates, 20 second ejaculates and 20 third ejaculates) at 20-minute intervals.

Semen was collected from each ram using an artificial vagina at temperatures ranging from 42 to 43 °C for each kind of collection frequency. All ejaculates were immersed in a 37 °C water bath immediately after collection. Each ram's sperm samples were evaluated for volume (ml), sperm concentration (10^9 spermatozoa/ml), mass motility (arbiter scale from 0 as immotile to 5 as vigorous cells) and individual motility (from 0 to 100 percent using a CASA system), viability, and abnormality using a UB203 microscope (400 x magnification).

Seminal plasma analysis

Seminal plasma from the five ram ejaculates was separated by centrifugation at 13000 rpm for 10 minutes at 4 °C after each collection, and the supernatants were pooled and kept at – 20 °C until analysis. The Lowry technique was used to determine the total protein concentrations (Lowry et al., 1951) based on bovine serum albumin (BSA) as standard. The total lipids were assessed according to the method of Woodman and Price (1972). Cholesterol concentrations were measured by a colorimetric method (Wybenga et al., 1970). Fructose

concentration (g / l) was evaluated according to the method of Mann (1948).

Semen conservation and quality analysis

A total of 250 microliters of each ram's ejaculate were pooled immediately after collection and first examination, then extended in skim milk to 0.8 10^9 spermatozoa/ml and assessed (T0). The extended semen was stored at 15 °C and evaluated after 8 and 24 h. Total (TM, %) and progressive (PM, %) motilities were calculated using a computer-assisted sperm motility analysis (CASA; ISAS, version 1.0.17, Proiser, Valencia, Spain). Sperm viability was determined by staining spermatozoa with nigrosine eosin under a microscope (400 x, light microscopy), and spermatozoa with partial or entire purple stain were regarded non–viable, while only spermatozoa with absolute exclusion of the stain were considered alive. The percentage of abnormal cells was assessed using a Diff–Quik staining (Automatic Diagnostic Systems S.L., Barcelona, Spain) and counted using a microscope (400 x, light microscopy). Sperm plasma membrane integrity was determined using the hypo-osmotic swelling test (HOST) (Revell, 2003). The percentage of spermatozoa with curled/swollen tail were counted among 200 sperm using a phase contrast microscope (400×, UB203 micro-scope). Lipid peroxidation (LPO) was determined in diluted sperm by measuring the amount of thiobarbituric acid reactive species (TBARS) formed (Allai et al., 2016).

Statistical analysis

Statistical analyses were performed using JMP SAS 11.0.0 (SAS Institute Inc., Cory, NC, USA) program. The data obtained were first tested for normality and homogeneity using the Shapiro–Wilk and Kolmogorov–Smirnov tests, respectively. The data of semen quality parameters seminal plasma composition and stored semen quality for each storage duration point were analyzed by a factorial design ANOVA. The statistical model included the fixed effect of ejaculation frequency (high frequency (HFE) *vs.* low frequency (LFE)) and subsequent ejaculates (first *vs.* second *vs.* third). When statistically significant differences were detected, the student's t test was applied to compare the means at $P<0.05$ for the effect of ejaculation frequency (high frequency (HFE) *vs.* low frequency (LFE)). Tukey's post hoc test was applied to compare the means at $P<0.05$ for the effect of subsequent ejaculates (first *vs.* second *vs.* third) and Data are expressed as mean ± SE. Correlations were calculated to establish the relationship between seminal plasma composition and chilled sperm quality after 24 hours of storage. Correlations were compared by means of Pearson's bivariate at $P<0.05$.

RESULTS

Effect of ejaculation frequency (HFE vs. LFE) on fresh sperm quality

The effects of ejaculation frequency and subsequent ejaculates on fresh sperm quality in INRA 180 rams are presented in Figure 1 and Table 1 and Table 2.

Ejaculation frequency had a significant effect on sperm volume, mass motility, abnormality, viability ($P<0.001$) and individual motility ($P<0.05$). Subsequent ejaculates significantly affected sperm volume, mass motility ($P<0.001$), individual motility ($P<0.05$), concentration ($P<0.01$) and abnormality ($P>0.05$).

Table 1. Effect of ejaculation frequency (Hight frequency (HFE; N= 90) *vs.* Low frequency (LFE; N = 60)) on fresh sperm quality and some seminal plasma components regardless of subsequent ejaculates in INRA 180 ram

Ejaculation frequency	HFE	LFE
Mass motility	3.33±0.09^b	4.33±0.08^a
Individual motility %	87.51±0.88^b	90.13±0.086^a
Viability %	84.44±1.21^b	90.25±1.01^a
Abnormality %	8.21±0.48^a	4.78±0.29^b
Total protein g/l	24.56±0.21^b	25.51±0.13^a
lipid g/l	3.50±0.07^b	3.79±0.03^a
Fructose g/l	5.07±0.16^b	5.61±0.08^a
Cholesterol g/l	1.42±0.05^b	1.61±0.07^a

a, b. Different superscripts within lines indicate an effect of ejaculation frequency ($P<0.05$)

The combination of ejaculation frequency and subsequent ejaculates had a significant impact on sperm volume (P<0.01), while the other parameters were not affected by this combination (P>0.05).

The first ejaculates recorded significantly the highest sperm volume, followed by the second and the third in the HFE. In the LFE, the first and second ejaculates showed the highest sperm volume compared to the third one. The three ejaculates in the LFE give the highest sperm volume compared to those in the HFE (Figure 1).

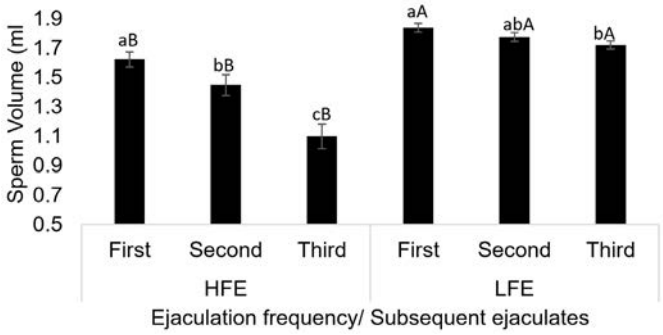

Figure 1. Effect of ejaculation frequency (high frequency (HFE) *vs.* low frequency (LFE)) and subsequent ejaculates (first *vs.* second *vs.* third) on sperm volume in INRA 180 ram

A, B. Different superscripts within bars indicate an effect of ejaculation frequency for each subsequent ejaculate (P<0.05).
a, b, c. Different superscripts within bars indicate an effect of subsequent ejaculates for each ejaculation frequency (P<0.05).

Sperm mass motility, viability (P<0.001) and individual motility (P<0.05) were higher in LFE compared to HFE (Table 1). While abnormality was higher in HFE compared to LFE (P<0.001). The first and second ejaculates recorded the highest sperm mass motility (P<0.001), individual motility (P<0.05) and concentration (P<0.01) and the lowest sperm abnormality (P<0.05) compared to the third one (Table 2).

Effect of ejaculation frequency (HFE vs. LFE) on ram seminal plasma composition

The effects of ejaculate frequency and subsequent ejaculates on seminal plasma (SP) composition in INRA 180 ram are presented in tables 1 and 2.

Ejaculation frequency had a significant effect on lipid, protein, fructose, and cholesterol concentrations in INRA 180 ram's seminal plasma (P<0.01). Subsequent ejaculates affected significantly the concentration of protein, fructose (P<0.01), cholesterol and lipid (P<0.05). The combination of ejaculation frequency and subsequent ejaculates had no significant impact on seminal plasma composition (P>0.05).

Total protein, lipid, fructose and cholesterol were significantly higher in LFE compared to HFE (Table 1). The first and second ejaculates recorded the greatest total protein, lipid, fructose, and cholesterol concentration in seminal plasma compared to the third ejaculate (Table 2).

Effect of ejaculation frequency (HFE vs. LFE) on stored ram sperm quality

The results of the effect of ejaculation frequency and subsequent ejaculates on stored sperm quality in INRA 180 ram are presented in Table 3 and Figure 2.

The ejaculation frequency (LFE *vs.* HFE) significantly affected the stored sperm quality parameters with different significant levels. At 0 h of storage, the ejaculation frequency had an effect on progressive motility (P<0.001) and membrane integrity (P<0.05) with the highest values recorded in LEF compared to HFE (Table 3). After 8 hours of storage, it had an effect on total motility (P<0.01), progressive motility, viability, and membrane integrity, with the highest values reported in LEF compared to HFE (Table 3). Besides, the results revealed that it had an effect on the abnormality (P<0.001), with the lowest levels reported in LEF compared to HFE (Table 3). At 24 h of storage, ejaculation frequency had a significant influence on progressive (P<0.001) and total motilities (P<0.05), as well as membrane integrity (P<0.01), with the highest values seen in LEF compared to HFE (Table 3), It also had an effect on the abnormalities (P<0.001), with the lowest levels reported in LEF compared to HFE (Table 3). Whatever the storage period was (0, 8, 24 h), the subsequent ejaculates (first *vs.* second *vs.* third ejaculates) had a significant influence on total and progressive motilities, viability, membrane integrity, and abnormalities (P<0.001). The first and second ejaculates had the highest total and progressive motilities, membrane integrity and viability, and the lowest abnormalities when compared to the third one (Table 3).

Table 2. Effect of subsequent ejaculates (First (N = 50) *vs.* second (N = 50) *vs.* third (N = 50)) on fresh sperm quality and some seminal plasma components (total protein, lipid, fructose and cholesterol) regardless of the frequency of ejaculation in INRA 180 ram

Subsequent ejaculates	First	Second	Third
Concentration (billion spz/ml)	3.61±0.08^{a}	3.42±0.08^{ab}	3.22±0.09^{b}
Mass motility	3.97±0.10^{a}	4.12±0.12^{a}	3.12±0.12^{b}
Individual motility %	90.75±0.69^{a}	90.58±1.03^{a}	84.35±1.22^{b}
Abnormality %	5.96±0.49^{b}	6.38±0.58^{ab}	8.18±0.67^{a}
Total protein g/l	25.30±0.22^{a}	25.17±0.29^{ab}	24.36±0.23^{b}
lipid g/l	3.75±0.07^{a}	3.63±0.08^{ab}	3.46±0.09^{b}
Fructose (g/l)	5.39±0.14^{a}	5.49±0.16^{a}	4.96±0.17^{b}
Cholesterol (g/l)	1.62±0.07^{a}	1.57±0.08^{a}	1.28±0.03^{b}

a, b. Different superscripts within lines indicate an effect of subsequent ejaculates (P<0.05)

The lipid peroxidation was the only parameter that was impacted by the ejaculation frequency, subsequent ejaculates, and their combination (P<0.001). At 0 h of storage, the HFE showed the highest lipid peroxidation compared to the LFE, whatever the subsequent ejaculate was. In HFE the third ejaculate recorded significantly higher lipid peroxidation compared to the first and second ones. However, in LFE, no significant lipid peroxidation was recorded between the three ejaculates (Figure 2). At 8 h, the lipid peroxidation was higher in HFE considering the first and third ejaculates, while no significant difference was recorded between HFE and LFE in the second ejaculate. In HFE, the third ejaculate recorded the highest lipid peroxidation compared to the first and second ones. However, in LFE, no significant lipid peroxidation was recorded between the three ejaculates (Figure 2). After 24 h of liquid storage, the HFE recorded the same lipid peroxidation in the first and second ejaculates as the LFE. However, the third ejaculate in HFE showed the highest lipid peroxidation than that in LFE. In HFE, the highest lipid peroxidation was recorded in the third ejaculate compared to the first and second ones. While in LFE, no significant difference was recorded between the three ejaculates in terms of lipid peroxidation (Figure 2).

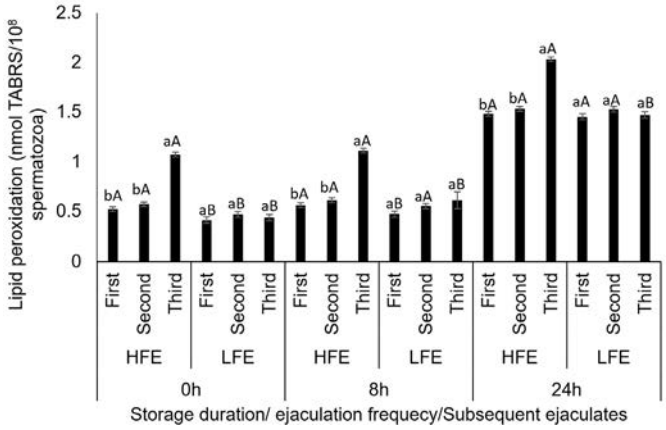

Figure 2. Effect of ejaculation frequency (high frequency (HFE) *vs.* low frequency (LFE)) and subsequent ejaculates (first *vs.* second *vs.* third) on stored sperm lipid peroxidation in INRA 180 ram

a, b, c. Different superscripts within bars indicate an effect of Subsequent ejaculates within each ejaculation frequency at each storage duration (P<0.05)

Correlation of seminal plasma components and chilled sperm quality after 24 h of storage

The results of the correlation test are presented in Table 4. It's shown that after 24 h of storage, progressive and total motilities and membrane integrity are positively correlated to total protein, lipid, and cholesterol (P<0.05). Viability was just positively correlated to cholesterol (P<0.05). Abnormality was negatively correlated to total protein, lipids, cholesterol, and fructose (P<0.05). Concerning, lipid peroxidation, it was negatively correlated to total protein and lipid (P<0.05).

Table 3. Effect of ejaculation frequency (high frequency (HFE) *vs.* low frequency (LFE)) and subsequent ejaculates (first *vs.* second *vs.* third) on stored sperm quality (Progressive and total motilities, viability, abnormality and membrane integrity) in INRA 180 ram

Storage period	Ejaculation frequency	Subsequent ejaculates	Progressive motility %	Total motility %	Viability %	Abnormality %	Membrane integrity %
0h	HFE	First	67.38±1.41^{aB}	89.17±1.44^{a}	90.50±1.48^{a}	6.81±0.56^{b}	85.50±1.48^{aB}
		Second	66.92±2.35^{aB}	91.83±1.54^{a}	93.17±1.58^{a}	7.67±0.79^{b}	88.17±1.58^{aB}
		Third	49.67±1.97^{bB}	85.88±1.33^{b}	87.17±1.35^{b}	11.56±0.69^{a}	82.17±1.35^{bB}
	LFE	First	73.75±0.99^{aA}	93.50±0.63^{a}	94.50±0.63^{a}	4.75±0.51^{b}	91.50±0.63^{aA}
		Second	76.81±1.87^{aA}	93.56±0.75^{a}	94.69±1.13^{a}	6.75±0.51^{b}	90.69±1.13^{aA}
		Third	57.33±2.15^{bA}	83.60±1.99^{b}	85.07±1.97^{b}	11.75±0.52^{a}	81.07±1.97^{bA}
8h	HFE	First	62±2.751.42^{aA}	84.25±1.59^{aA}	85.25±1.59^{Aa}	9.92±0.54^{bA}	80.25±1.59^{aA}
		Second	59.08±3.19^{aA}	83.08±2.27^{aA}	84.08±2.27^{aA}	10.67±0.79^{bA}	79.08±2.27^{aA}
		Third	42.79±2.34^{bA}	77.50±1.79^{bA}	78.50±1.79^{bA}	14.56±0.69^{aA}	73.50±1.79^{bA}
	LFE	First	70.63±1.34^{aB}	89.94±0.72^{aB}	90.94±0.72^{aB}	5.28±0.49^{bB}	87.94±0.72^{aB}
		Second	73.19±1.94^{aB}	90.31±1.40^{aB}	92.88±1.38^{aB}	7.28±0.49^{bB}	88.88±1.38^{aB}
		Third	47.93±2.64^{bB}	79.60±2.56^{bB}	81.87±2.61^{bB}	12.28±0.50^{aB}	77.87±2.61^{bB}
24h	HFE	First	49.13±2.31^{aB}	77.08±2.14^{aB}	78.42±2.06^{a}	13.92±0.60^{bA}	73.42±2.61^{aB}
		Second	40.33±2.49^{bB}	70.21±3.17^{bB}	71.54±3.15^{ab}	14.67±0.79^{bA}	66.54±3.15^{bB}
		Third	34.91±2.72^{cB}	67.22±2.69^{bB}	68.56±2.75^{b}	19.56±0.69^{aA}	63.79±2.64^{bB}
	LFE	First	59.25±1.31^{aA}	83.25±1.42^{aA}	84.25±1.42^{a}	5.28±0.50^{bB}	81.25±1.42^{aA}
		Second	48.38±2.14^{bA}	76.69±2.02^{bA}	78.94±2.07^{ab}	7.28±0.51^{bB}	74.94±2.07^{bA}
		Third	38.60±2.49^{cA}	69.53±3.56^{bA}	67.31±5.51^{b}	14.69±1.39^{aB}	67.67±3.60^{bA}

a, b, c. Different superscripts within lines indicate an effect of Subsequent ejaculates within each ejaculation frequency at each storage duration (P<0.05)

A, B. Different superscripts within lines indicate an effect of ejaculation frequency within each Subsequent ejaculate at each storage duration (P<0.05)

Table 4. Correlation coefficients between seminal plasma composition and chilled semen quality after 24 hours of liquid storage in INRA 180 rams

	Total protein	Total lipid	Cholesterol	Fructose	PM	TM	VIAB	ABN	MI	LP
Total protein	1	0.97*	0.90*	0.98*	0.85*	0.85*	0.81	-0.98*	0.88*	-0.74*
Total lipid		1	0.83*	0.92*	0.88*	0.88*	0.79	-0.96*	0.93*	-0.79*
Cholesterol			1	0.87*	0.89*	0.89*	0.94*	-0.93*	0.90*	-0.50
Fructose				1	0.74	0.73	0.71	-0.95*	0.79	-0.71
PM					1	0.42*	0.38*	-0.52*	0.43*	-0.37*
TM						1	0.80*	-0.34*	0.82*	-0.28*
VIAB							1	-0.37*	0.99*	-0.20*
ABN								1	-0.32*	0.51*
MI									1	-0.28*
LP										1

* Significant at $P<0.05$; PM: Progressive motility (%); TM: Total motility (%); VIAB: Viability (%); ABN (Abnormality) (%); MI: Membrane integrity (%); LP: (Lipid peroxidation) (nmol TABRS/108 spermatozoa); Total protein (g/l); Lipid (g/l); Cholesterol (g/l); Fructose (g/l)

DISCUSSION

A previous study demonstrated that the INRA 180 rams can generate sperm of high quality over the whole year (Benmoula et al., 2017). However, in order to extensively use males of high genetic value for natural breeding or as sperm donors for genetic improvement in artificial insemination centers, it was necessary to investigate the effect of collection frequency on sperm output and quality before and after conservation.

The effect of semen collection frequency upon sperm quality in domestic animals was reported by other investigators on rams, bulls, boars, and stallions (Wybenga et al., 1970; Foote, 1978; Salamon and Maxwell, 1995; Ollero et al., 1996; Kaya et al., 2002; Revell, 2003; Yotov et., 2011). However, to the best of our knowledge, this is the first study to report the influence of ejaculation frequency on protein, lipid, cholesterol, and fructose concentrations in seminal plasma and their correlation to sperm ram liquid storage. In this study the fresh sperm quality parameters were higher in LFE compared the HFE. Besides, in both kind of collection frequencies; the first and the second ejaculates showed the highest sperm quality compared to the third one. These findings are consistent with previous research demonstrating that collecting numerous ejaculates dramatically decreased sperm quality in rams, and the degree of this loss varied with the duration of abstinence and the duration between ejaculation (Yotov et al., 2011). In fact, it has been observed that a one-week rest time (LFE) between each collection had less negative effects on sperm quality than a three-day rest period (HFE) (Nahak et al., 2015). In other words, when several ejaculates are necessary, the data presented in this study suggests that a 1-week time period is sufficient for semen quality restoration in INRA 180 rams. On the other hand, a 3-day time scale can only ensure a partial return of this quality. These effects might simply be the result of sperm reserve depletion (Al-Bulushi et al., 2018).

Concerning the ejaculation duration, 20 minutes was chosen based on our previous experiments. In fact, this time is sufficient for a technician to collect, examine, and store sperm from each single ram. Besides, this could explain the disparity between our findings and those of Yotov et al. (2011), who chose 10 minutes between ejaculates and discovered that the second ejaculate produced the best results.

As it was indicated in fresh sperm quality, seminal plasma could be also affected by the frequency of collection (Kaya et al., 2002). Our results showed that total protein, lipid, fructose and cholesterol have more or less the same tendency recorded for the fresh sperm quality parameters. They were significantly higher in LFE compared to HFE. Besides, the first and second ejaculates recorded the greatest concentrations in SP compared to the third ejaculate. The reasons why there is more protein, lipid, cholesterol and fructose in seminal plasma in LFE than HFE and in the first and second ejaculates than in third are not known. Probably it came from either the epididymal fluid or the spermatozoa or, more likely, from both.

In the present study the sperm stored quality was substantially higher in LFE than in HFE. In addition, the first ejaculate had the highest sperm quality parameters, followed by the second one. On the other hand, the third ejaculate, had the lowest sperm quality characteristics. This finding was related to seminal plasma composition (Amiridis et al., 2005). In fact, protein, lipid, and cholesterol, concentrations have a substantial influence on sperm preservation (Druart et al., 2009; Benmoula et al., 2017; Badi et al., 2018). Theoretically, this could be owing to seminal plasma antioxidants being depleted after several ejaculations (Sarlós et al., 2005; Allai et al., 2018; Tvrdá et al., 2011). Especially since, in our study, lipid peroxidation of chilled sperm was negatively correlated to total protein and lipid concentrations in seminal plasma. Indeed, Marti et al. (2007), demonstrated that, antioxidant enzyme activities (superoxide dismutase and catalase) were higher in the first ejaculates compared to the other ones. Besides, it is documented that a decrease in polyunsaturated fatty acids and docosahexaenoic acid in seminal is related to sperm oxidation (Argov-Argaman et al., 2013).

In our investigation, sperm motility, viability, and membrane integrity were all highly linked with seminal plasma protein content. In other investigations, it was well documented that total protein concentration in rams' seminal plasma was strongly correlated to semen characteristics and stored ram semen quality (Almadaly et al., 2016; Benmoula et al., 2017; Badi et al., 2018). In particular, seminal plasma proteins prevent sperm alterations due to refrigeration and apoptosis (Mendoza et al., 2013). Besides, Soleilhavoup et al. (2014) showed that several sperm membrane proteins interacting with the cytoskeleton, glycolysis enzymes and sperm-associated proteins correlated with better liquid storage. Concerning lipid and cholesterol they were correlated to sperm motility, viability and membrane integrity in this study. Similarly, Masoudi et al. (2016) revealed that lipid and cholesterol concentration in seminal plasma are associated with membrane fluidity, acrosome reaction, sperm motility and viability. In fact, polyunsaturated fatty acid, provide suitable stabilization by maintaining liquidity and elasticity of cell membrane, which lead to increase the sperm cell number and motility by decreasing lipid peroxidation (Tvrdá et al., 2011; Towhidi et al., 2013).

The obtained results strongly support the possibility to intensively used rams with high genetic merit while throwing light on the problem to obtain high-quality semen and thus higher fertilization rates when multiple ejaculates are to be used in artificial insemination.

CONCLUSION

In conclusion, the frequency of ejaculation affects the quality of fresh sperm and all parameters related to the survival rates of stored semen from INRA 180 rams, with the highest sperm quality in the first and second ejaculates. For the studied breed, it has been shown that the semen from 3 consecutive ejaculates collected at 20-min intervals with 1 week of abstinence is suitable for artificial insemination immediately after collection and dilution. Also, when storage and transportation are required, the first and second ejaculates could be the most appropriate. Future field trials are probably needed to reveal the effect of ejaculate frequency and the time of storage on conception rates.

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