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Post-thaw survival and acrosome integrity of spermatozoa of Lecce ram frozen in different seasons with a milk-egg yolk extender

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ABSTRACT

The influence of the period of semen collection on post-thawing survival, motility and acrosome integrity of spermatozoa in Lecce ram was studied throughout an entire year. The year was divided into the seasons: winter and spring (first semester) and summer and autumn (second semester). Semen from 5 adult rams was collected every two weeks by artificial vagina and frozen according to a freezing system based on milk-lactose egg yolk to constitute semen doses of 400×10^6 spermatozoa. At thawing, survival and acrosomal status of cells were assessed and the motility of the sperm and their kinetic rating (scale 0 to 5 score) were determined at thawing (0 h), and after 1 and 3 h incubation (37°C). Semen collected during the first semester (winter – spring) of the year showed the highest ($P < 0.01$) proportion of post-thaw live spermatozoa, with the maximum value in winter ($P < 0.01$), and the best acrosomal status of spermatozoa, considered as both total proportion of spermatozoa with acrosome break down and spermatozoa without acrosomes. Acrosome integrity was positively correlated ($r = 0.32$; $P < 0.01$) with post-thaw sperm viability. Motility of spermatozoa at thawing was not influenced by the period of semen collection. However, after 3 h incubation sperm motility was higher ($P < 0.01$) during the first semester of the year, without a difference between winter and spring. A marked individual ram effect was found on freezability of semen. The results provide evidence that the period of semen collection can influence freezability of spermatozoa in Lecce ram. The best characteristics of spermatozoa were observed during the first semester of the year, corresponding to the sexual hypoactivity season for this breed.

Key Words: Sheep-male reproduction, Seasonal dynamics, Cryopreservation.

RIASSUNTO

CARATTERISTICHE POST-SCONGELAMENTO DEGLI SPERMATOZOI DI ARIETI DI RAZZA LECCESE CONGELATI IN STAGIONI DIFFERENTI CON L'IMPIEGO DI UN MESTRUO A BASE DI LATTE E TUORLO D'UOVO

Lo studio, condotto per un intero anno, ha avuto lo scopo di valutare l'influenza della stagione di raccolta del seme sulla sopravvivenza post-scongelo, sulla motilità e sull'integrità acrosomiale degli spermatozoi di arieti di razza Lecce. Sono state considerate le stagioni: inverno e primavera (primo semestre) ed estate e autunno (secondo semestre). Cinque arieti adulti sono stati sottoposti alla raccolta del seme, mediante vagina artificiale. Il seme è stato sottoposto a congelamento utilizzando un mestruo diluitore a base di latte e tuorlo d'uovo e sono state costituite dosi da 400×10^6 spermatozoi. Allo scongelamento è stata valutata la sopravvivenza e lo status acrosomiale degli spermatozoi, mediante doppia fluorocromatizzazione (Hoechst 33258 e FITC-PSA). La percentuale di spermatozoi mobili e la motilità individua-

le degli spermatozoi (motilità progressiva; scala 0 - 5) è stata valutata allo scongelamento (0 ore), dopo 1 e 3 ore di incubazione (37°C).

Il seme raccolto durante il primo semestre dell'anno (inverno-primavera) ha evidenziato la più elevata ($P < 0,01$) percentuale di spermatozoi vivi allo scongelamento, con il valore massimo in inverno ($P < 0,01$). Anche lo status acrosomiale, considerato come percentuale totale di spermatozoi con acrosoma danneggiato e spermatozoi privi di acrosoma, è risultato migliore ($P < 0,01$) durante il primo semestre. L'integrità acrosomiale è risultata positivamente correlata ($r = 0,32$; $P < 0,01$) con la vitalità post-scongelamento degli spermatozoi.

La motilità allo scongelamento non è stata influenzata dalla stagione di raccolta del seme. Tuttavia, dopo l'incubazione per 3 ore, la motilità è stata maggiore ($P < 0,01$) durante il primo semestre dell'anno, senza differenza fra l'inverno e la primavera. Sussiste una marcata variabilità individuale per la congelabilità del seme.

I risultati hanno evidenziato che la stagione di raccolta del seme influenza la congelabilità degli spermatozoi negli arieti di razza Leccese. Le migliori caratteristiche di congelabilità degli spermatozoi sono state osservate durante il primo semestre dell'anno, in corrispondenza della stagione di ipoattività sessuale per questa razza.

Parole chiave: Caratteristiche spermatozoi, Variazioni stagionali, Congelamento, Ariete.

Introduction

The remarkable success obtained in the cryopreservation of spermatozoa in bull semen is not applicable to other species of productive interest, such as sheep, for example. It has been reported that there are two main causes for different results between the species: (i) the physiology and biochemistry of the spermatozoa, and (ii) different characteristics in the anatomy and physiology of sperm transport in the female tract (Holt, 2000a). In ewes, the anatomical conformation of the cervix blocks catheter insertion for the direct intrauterine deposition of semen. Thus, cervical insemination is generally performed on the posterior vagina and it requires semen doses with high sperm concentration, that is large numbers of spermatozoa in a small volume (Allison and Robinson, 1971; Salamon, 1977; Colas, 1983). Cervical insemination using frozen-thawed spermatozoa generally results in low fertility (Colas, 1983; Maxwell and Watson, 1996; Salamon and Maxwell, 2000; Yoshida, 2000). It is well known that cryopreservation techniques cause extensive sperm mortality through the processes of cooling, freezing and thawing (Watson, 1995, 2000; Salamon and Maxwell, 2000). For this, the quality of spermatozoa and their aptitude to the freezing process assume a critical importance and a lower tolerance of poor sperm is required in the collection of semen which have to be frozen, in order to obtain success from AI.

Several studies on rams in temperate zones have demonstrated seasonal variations that affect reproductive activity (Poulton and Robinson, 1987;

Martin *et al.*, 1990; Perez *et al.*, 1997; Martemucci *et al.*, 2000), as well as freezability of spermatozoa (Maxwell, 1980; Curnock *et al.*, 1984; D'Alessandro and Martemucci, 2003) and their fertilizing ability (Colas and Brice, 1976; Guerin *et al.*, 1992).

This study was conducted on Leccese rams, an autochthonous breed, to investigate the effects of the period of semen collection on post-freezing survival and acrosome integrity of spermatozoa frozen in straws with a milk-lactose-egg yolk extender, in order to establish the optimal season of freezing semen to be employed in AI.

Material and methods

Collection, diluent and storage of semen

Semen was collected for a period of one year from 5 adult Leccese rams reared in semi-intensive conditions on the farm of the Faculty of Agricultural Science, Bari University (Southern Italy, 41°N latitude). The rams were subjected to a rhythm of semen collection twice a week by artificial vagina and assessment of the quantitative and qualitative characteristics of the semen from each ram were evaluated every two weeks.

Only ejaculates of good quality (sperm concentration $\geq 3 \times 10^9$ spermatozoa / mL; motility score $> 4/5$ of a scale from 1 to 5; proportion of motile cells $> 60\%$) were processed for freezing.

For the study, semen was frozen according to a freezing method based on the use of milk - lactose egg yolk extender (Colas, 1975). Briefly, semen was pre-diluted (1:1, semen : diluent) and then diluted to a concentration of 1.5×10^9 spermatozoa / mL with the unglycerolate diluent containing lactose

(8.24%, wt/v) and egg yolk (20%, v/v) (pH = 6.65 to 6.70; 450 mOsm). After 2 h of gradual cooling to 4°C, the glycerolate diluent - made of skim milk (14%, wt/v), sulphanilamide (17.4 mM), trisodium citrate (x 5.5 H₂O, 6 mM), glycerol (10%, v/v) and antibiotics - was added in a volume equal to 2 / 5 of the final volume to achieve a sperm concentration of 900 x 10⁶ spermatozoa / mL. Thus, semen was loaded into 0.5 mL French straws (IMV) constituting doses of 400 x 10⁶ spermatozoa / straw, which were frozen for 7 min in nitrogen vapor (-75°C) and then stored in liquid nitrogen (-196°C) until used.

Assessment of survival and acrosome integrity

The straws were thawed in a water bath (37°C) for 30 sec.

The assessment was performed by dual staining with Hoechst 33258 (Sigma; Milan, Italy) and fluorescein isothiocyanate labelled *Pisum Sativum* Agglutinin (FITC - PSA, Sigma), as described by Cross *et al.* (1986), with some modifications (D'Alessandro and Martemucci, 2003). Hoechst 33258 is a fluorescent dye which stains the dead cells blue, binding to DNA, while FITC-PSA binds to glycoproteins of acrosome, and stains sperm acrosome green, with variable colour intensity depending on the level of acrosome integrity. Spermatozoa with acrosome breakdown were classified as: sperm without acrosome (cells completely unstained), sperm with damaged acrosome (cells with patchy fluorescence) and unviable sperm with widespread fluorescence (cells completely fluorescing: head, post-acrosomial region and principal piece of tail or whole tail). Acrosome intact sperm appear with the acrosome cap fluorescing (bright green). Total spermatozoa with acrosome breakdown, including all forms of acrosome breakdown, was also reported.

Assessment of motility

Sperm motility was evaluated as proportion of motile spermatozoa and motility score, under a microscope fitted to a warm stage (37°C). For the assessment, the samples were transferred to phosphate buffered saline (PBS) and motility was evaluated at 0 h (thawing) and after 1 and 3 h incubation (37°C), maintaining the semen in PBS for the incubation period.

The proportion of motile sperm was determined on 40 cells of the Makler counting chamber under a phase contrast microscope (x 200) fitted with a stage warmer at 37°C. Motile spermatozoa were determined as the difference between the total number of spermatozoa and the immotile spermatozoa, with the aid of a Polaroid freeze-frame video recorder connected to a microscope (D'Alessandro and Martemucci, 2003). Motility score was determined microscopically (x 400) with a drop of diluted semen (1:100 in PBS), at thawing, after 1 h and again after 3 h incubation (37°C) period using the scale 0 to 5 (0: no motion; 5: very rapid forward motion).

The experimental data were referred as: seasonal mean values, where season was the months of January - February - March as Winter, April - May - June as Spring, July - August - September as Summer and October - November - December as Autumn; means of both the first semester of the year, from January to June, and the second semester, from July to December.

Statistical analyses

Prior to statistical analysis, the data were tested for homogeneity of variance by the Bartlett's test and, where necessary, transformed (log + 1). Data were analysed by least squares analysis of variance using the GLM procedure of the SAS system (SAS, 1987). The statistical model used considered the effects of season, month of semen collection, animal and their interactions. Least squares means between seasons were compared by the T value (PDIFF option), while a comparison between semesters was made by linear contrast. Correlation between the different parameters were also determined by regression analysis (SAS, 1987).

Results

The period of semen collection significantly influenced (P<0.01) post-thaw survival of the spermatozoa. The proportion post-thaw live spermatozoa was higher (P<0.01) when semen was collected in winter and spring and this corresponded to a higher (P<0.01) post-thaw survival rate of sperm during the first semester (Table 1).

Table 1. Effects of the period of semen collection on the post-freezing live spermatozoa (mean \pm SE).

Period	Live sperm (%)
Winter	48.2 \pm 1.6 ^A
Spring	42.2 \pm 1.6 ^C
Summer	36.2 \pm 1.6 ^B
Autumn	40.3 \pm 1.7 ^{BC}
1 st Semester ¹	45.2 \pm 1.3 ^D
2 nd Semester ²	38.3 \pm 1.4 ^E

¹ January to June

² July to December

^{A, B, C} Significant differences among the seasons ($P < 0.01$)

^{D, E} Differences between the two semesters ($P < 0.01$)

The detrimental effect of the second semester of the year on sperm viability was most evident in summer (Table 1). The ram effect was significant ($P < 0.05$) with respect to the survival of spermatozoa at thawing.

Total proportion of spermatozoa with acrosome breakdown was significantly ($P < 0.01$) influenced by the season of semen collection and individual ram effect. The lowest ($P < 0.01$) incidences of acrosomal breakdown were observed in semen collected during the first semester, and particularly in spring ($P < 0.01$) (Table 2).

The proportion of spermatozoa with intact acrosomes at thawing resulted positively correlated ($P < 0.01$) to proportions of post-thaw live sperm ($r = 0.32$).

Also the proportion of spermatozoa without acrosomes was significantly ($P < 0.01$) influenced by the season of semen collection and ram effect. The incidence of spermatozoa without acrosome was higher in the second semester ($P < 0.01$), with the highest occurrence in summer ($P < 0.01$) (Table 2).

The proportion of spermatozoa with acrosomal damage was influenced ($P < 0.01$) by the month of collection, but the effect of season was not significant. There were no differences in proportions of sperm with acrosomal damage between the two

semesters and values were similar for all seasons. The proportions of sperm without acrosome and sperm with damaged acrosome were positively correlated ($r = 0.30$; $P < 0.01$) only when considering the entire year.

Of the total acrosome abnormalities, the proportion showing widespread FITC-PSA fluorescence was the least influential, with values ranging between 0.1% (winter) to 0.8% (spring and autumn; $P < 0.01$).

The motility of sperm was not influenced by the season of semen collection, but the effect of the individual was significant ($P < 0.01$) after 3 h of incubation. The proportion of motile sperm, ranged from 35.1 to 38.1% at thawing, and decreased with time of incubation (Table 3). After 3 h of incubation sperm motility during the first semester of the year was higher ($P < 0.01$), with similar values in winter and spring ($P < 0.01$) (Table 3).

The proportion of motile sperm after 3 h of incubation was negatively correlated with sperm motility at thawing ($r = -0.27$; $P < 0.05$).

The motility score of sperm followed the same trend as motility (Table 4). After 3 h of incubation, motility score (scale 0 to 5) ranged from 3.04 to 3.26 for the different seasons.

Table 2. Effects of the period of semen collection on the post-freezing acrosome status of spermatozoa (mean \pm SE).

Period	Total spermatozoa with acrosome breakdown (%)	Spermatozoa without acrosome (%)	Spermatozoa with damage of acrosome (%)	Unviable spermatozoa with widespread fluorescence (%)
Winter	69.5 \pm 2.0 ^{BC}	28.2 \pm 1.4 ^B	40.9 \pm 2.1	0.1 \pm 0.1 ^A
Spring	65.3 \pm 1.9 ^C	29.3 \pm 1.4 ^B	35.1 \pm 2.1	0.8 \pm 0.1 ^B
Summer	85.1 \pm 2.0 ^A	44.1 \pm 1.4 ^A	40.4 \pm 2.2	0.5 \pm 0.1
Autumn	74.3 \pm 2.1 ^B	34.3 \pm 1.5 ^C	39.1 \pm 2.3	0.8 \pm 0.1 ^B
1 st Semester ¹	67.4 \pm 1.4 ^D	28.8 \pm 1.2 ^D	38.0 \pm 1.5	0.5 \pm 0.07
2 nd Semester ²	79.7 \pm 1.5 ^E	39.2 \pm 1.3 ^E	39.7 \pm 1.6	0.7 \pm 0.08

¹ January to June

² July to December

^{A, B, C} Significant differences among the seasons ($P < 0.01$)

^{D, E} Differences between the two semesters ($P < 0.01$)

Discussion

A large number of techniques have been tested in order to reduce or stop the metabolism of spermatozoa, thereby prolonging their fertile life. This is commonly achieved by cryopreservation (Maxwell and Salamon, 1993) and different extenders have been tested to freeze ram semen in an attempt to minimize detrimental effects of the freezing process on sperm (Salamon and Maxwell, 1995a; 2000). The main physiological and functional changes in spermatozoa which occur include an irreversible reduction in viability, acrosome integrity, motility and thus, fertility (Salamon and Maxwell, 2000; Medeiros *et al.*, 2002). The sperm capability to resist cold shock, generally known as freezability, is an important requirement to maintain the plasma and outer acrosomal membranes intact and undamaged throughout cryopreservation and thawing. There is evidence that freezability of sperm is affected by season within breeds (Maxwell, 1980; Curnock *et al.*, 1984).

It has been established that the optimal conditions for semen freezing may differ with the osmotic pressure, composition of extenders and

the dilution rate of the semen (Ritar and Ball, 1993; D'Alessandro *et al.*, 2001a; El-Alamy and Foote, 2001). Previous studies performed on Leccese rams have evaluated seasonal variations in the quality of fresh semen (D'Alessandro *et al.*, 2001a) and semen freezability using a freezing method based on Tris – fructose egg yolk extender to constitute semen doses of 100×10^6 sperm (D'Alessandro and Martemucci, 2003). In the present study the seasonal variation in the freezability of spermatozoa using a freezing system based on a milk – lactose egg yolk as extender was used (Colas, 1975) to constitute semen doses of 400×10^6 spermatozoa suitable for cervical inseminations.

The results of this experiment indicate that spermatozoa ejaculated during winter and spring (corresponding to the non-breeding season; Martemucci *et al.*, 1979), have a higher ability to survive freezing and a better acrosomal integrity than semen frozen during the second semester. Contrary to these findings, in fresh semen a higher survival rate of sperm was observed during the second semester (D'Alessandro *et al.*, 2001b) due to the positive effect of decreasing photoperiod on

Table 3. Effects of the period of semen collection on the percentage of motile spermatozoa at thawing (0 h) and during the post-thawing incubation (37 °C) for 1 and 3 h (mean \pm SE).

Period	Motile spermatozoa (%) after incubation for		
	0 h	1 h	3 h
Winter	35.1 \pm 2.7	24.2 \pm 2.3	14.6 \pm 1.3 ^A
Spring	35.1 \pm 2.3	22.9 \pm 2.0 ^b	14.3 \pm 1.1 ^A
Summer	33.9 \pm 2.3	22.8 \pm 2.0 ^b	9.6 \pm 1.1 ^B
Autumn	38.1 \pm 2.5	29.2 \pm 2.1 ^a	12.6 \pm 1.2
1 st Semester ¹	35.1 \pm 2.6	23.6 \pm 1.7	14.5 \pm 1.0 ^D
2 nd Semester ²	36.9 \pm 2.1	26.0 \pm 1.6	11.1 \pm 0.9 ^E

¹ January to June.

² July to December

^{A, B; a, b} Significant differences among the seasons (A - B: $P < 0.01$; a - b: $P < 0.05$)

^{D, E} Differences between the two semesters ($P < 0.01$)

Table 4. Effects of the period of semen collection on the post-freezing kinetic rating (scale 0 to 5) of spermatozoa at thawing (0 h) and after 1 and 3 h incubation (37 °C) (mean \pm SE).

Period	Kinetic rating after incubation for		
	0 h	1 h	3 h
Winter	4.17 \pm 0.10	3.7 \pm 0.2	3.02 \pm 0.20
Spring	4.15 \pm 0.09	3.9 \pm 0.1	3.26 \pm 0.16
Summer	4.06 \pm 0.09	3.7 \pm 0.1	3.04 \pm 0.16
Autumn	4.23 \pm 0.09	3.9 \pm 0.2	3.24 \pm 0.17
1 st Semester ¹	4.16 \pm 0.08	3.8 \pm 0.1	3.14 \pm 0.14
2 nd Semester ²	4.14 \pm 0.08	3.8 \pm 0.1	3.14 \pm 0.13

¹ January to June

² July to December

sexual activity of rams (Colas, 1980; Mickelsen *et al.*, 1981; Dufour *et al.*, 1984). This pattern has also been observed in frozen semen processed with a Tris – fructose egg yolk extender (D'Alessandro and Martemucci, 2003). No explanation can be offered for these findings. It is possible that specific components of the milk – lactose egg yolk extender and their concentrations have preserved the plasmatic membrane integrity, index of sperm viability, as well as the acrosomal membrane. It was reported that milk diluents contain several compounds which act positively on spermatozoa viability (Salamon and Maxwell, 1995a). In the extender used in this study the proportion of egg yolk ranged from 2.2 to 10.2%. The protective effect of egg yolk has been attributed to the low protein density fraction, specifically the phospholipids, a high molecular weight component, which act at the cell surface (Watson, 1975). Loss of phospholipids from sperm membrane is assumed to occur during cold shock (Parks and Graham, 1992). In addition, it has been reported that egg yolk also protects against lipid peroxidation (Marwell and Stojanov, 1996), induced by cryopreservation (Alvarez and Storey, 1992; Bell *et al.*, 1993; O'Flaherty *et al.*, 1997) thus acting as an antioxidant. Furthermore, there is the possibility that the positive effects due to addition of milk lipoproteins enhance those of the egg yolk on the sperm surface. All this may compensate for the lower amount of proteins in sperm membranes attributed to the adverse breeding season (Smith *et al.*, 1999) combined with the loss due to the freezing process (Centola *et al.*, 1990; Ollero *et al.*, 1998), probably acting on the distribution and/or interaction of lipids and proteins within the plasma membrane. It must be taken into account that cryoresistance is related to the composition of the plasma membrane (Parks and Graham, 1992) and that acrosomal plasma membranes may differ in their composition (Holt and North, 1985). The positive correlation between post-thawing viability and acrosomal integrity of the sperm confirmed what was reported in previous studies (Valcarcel *et al.*, 1997; D'Alessandro and Martemucci, 2003).

Considering the different classes of the acrosome status in sperm, the results indicate a higher incidence of acrosome damage, corresponding to the patchy FITC-PSA fluorescence. The patchy flu-

orescence of the acrosome membrane is indicated as acrosome reaction in progress (Sukardi *et al.*, 1997). The lack of differences among the seasons could demonstrate that there is an effect due only to the cryopreservation process, which induces a status similar to capacitation and acrosome reaction (Watson, 1995, 1996; Maxwell and Watson, 1996). The loss of acrosome, index of complete sperm capacitation (Sukardi *et al.*, 1997) and shorter life of spermatozoa (Watson, 1995, 1996; Maxwell and Watson, 1996), was highest in summer, the season in which the spermatozoa show the lowest qualitative characteristics. Sperm with widespread fluorescence in the whole cell was very low (< 1%). This form of staining can be due to the effect of a long permeabilization of cellular membrane during the staining procedure, which causes loss in bond selectivity of PSA for the acrosomal components, or to a higher availability for the PSA of non acrosomal bond sites in dead cells (Mendoza *et al.*, 1992).

Motility of spermatozoa at thawing was not affected by the period of semen collection at thawing, while after 3 h incubation the negative influence of the second semester, especially in summer, was evident. It is possible to conclude that the extender used in the freezing process could be adequate to compensate for the different qualitative sperm characteristics induced by the period of the year (D'Alessandro *et al.*, 2001a). Incubation highlights different modifications of cell structures and functions connected with the motility of sperm. It is noteworthy that in a previous study (D'Alessandro and Martemucci, 2003) on the same Leccese rams, using a Tris – fructose egg yolk extender (final concentration of 100×10^6 spermatozoa), motility of spermatozoa was lower than that obtained in the present study with a milk-lactose-egg yolk-based extender. These findings are in agreement with the studies of D'Alessandro *et al.* (2001b) where the same milk - lactose egg yolk extender displayed a better protective effect on sperm motility after thawing, compared to the Tris - fructose egg yolk extender at the pre-freezing sperm concentrations between 50 to 400×10^6 spermatozoa/mL (inseminate doses of 10 to 80×10^6 spermatozoa). This could be attributed to the higher osmolarity of the extender (450 vs 300 to 326 mOsm, milk vs Tris based extender), which in

buck semen has been linked to the better tolerance of spermatozoa at all dilution rates compared to hypotonic diluents (Ritar and Ball, 1993).

The results of this study also confirm the inter-individual differences in sperm freezability found in other research (Maxwell, 1986; Butler and Maxwell, 1988; Eppleston and Maxwell, 1991) which can be attributed to both genetic and environmental conditions (Salamon and Maxwell, 1985b; Holt, 2000b).

In conclusion, the results indicate that period of semen collection influences freezability of spermatozoa in Leccese rams. The post-freezing characteristics of sperm are higher during the first semester of the year (winter – spring), which in the experimental conditions coincides with the period of sexual hypoactivity.

Conclusions

The results show that in Leccese rams the period of semen collection influence freezability of spermatozoa. Overall, the best freezability characteristics of spermatozoa, using milk-lactose-egg yolk extender, occur during the first semester of the year (winter to spring), corresponding to the sexual hypoactivity season for this breed.

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