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Sperm Abnormalities in Post-thawed Semen of Tunisian Arab Stallions

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Abstract: The study was undertaken in order to evaluate sperm morphology features of post-thawed semen of Tunisian Arab stallions. Forty two ejaculates was collected and frozen, during years 2009 and 2010, from 9 stallions aged between 9 to 24 years. After thawing, sperm morphology was studied after eosin-nigrosin stain. The percentages of abnormal head, mid piece, flagella, sperm with droplets and the total abnormal sperm were determined. Analysis of variance was carried out using SAS software. The results showed that all sperm morphology features varied among ejaculates within stallion and among stallions ($p<0.01$). The percentage of abnormal flagella and total abnormal sperm varied between young and old stallions ($p<0.01$). We concluded that freezing and thawing processes increased abnormal sperm that is due to cell alteration for old and young stallions, and consequently, the decrease of the quality of the thawed semen of Tunisian Arab stallions.

Key words: Morpholog, post-thawed, semen, Arab, stallions

INTRODUCTION

In all species, the evaluation of sperm morphology is an important step to understand reasons for decreasing fertility (Garavance *et al*., 1997). In human, abnormal sperm morphology increases low fertility (Chan *et al*., 1989). In stallions, controversially, some studies reported no relation between sperm morphology features and fertility (Voss *et al*., 1981; Love *et al*., 2000), others however showed that an increase in sperm abnormality affected negatively stallion's fertility (Jasko *et al*., 1990). For example, it was reported that presence of proximal droplets affected negatively pregnancy rate per cycle (Jasko *et al*., 1990). Besides, Katila (2001) showed that low number of alive and normal post-thaw sperm could decrease the pregnancy rate. The objective of this study was to investigate the sperm morphology features of post-thawed semen of Tunisian Arab Stallion and to determine factors that could affect this morphologic quality.

MATERIAL AND METHODS

General: The study took place in the National Stud Farm of Sidi Thabet located in the north of Tunisia. Forty two ejaculates from 9 Tunisian Arab stallions aged between 9 to 24 years (young stallions: age <15 years, n = 5; old stallions: age = 15 years, n = 4) were used (2 to 10 ejaculates from each stallion). Semen of these stallions were collected and frozen during the month of December 2009 and December 2010. The freezing semen was performed when the percentage of mobile spermatozoa, determined under light microscope, and sperm concentration were respectively greater than 70% and 120 millions spermatozoa mL. The freezing semen was carried out according to the French method (Haras Nationaux, 2004): Once filtered, the semen was immediately diluted in the UHT skimmed milk extender supplemented with antibiotics gentamicin (50 mg L^{-1}) and penicillin (50000 IU L^{-1}) (¼ of semen for ¾ of the extender) in a water bath at 37°C. Then, the semen was placed in a water bath at 22°C for 10 min. After that, the semen was centrifuged during 10 min at 3000 rpm (600 g). The supernatant was removed and the pellet of sperm was re-suspended in the INRA 96® (IMV, L'Aigle, France) supplemented with 2% chicken egg yolk clarified and 2.5% glycerol to obtain a final concentration of 100 millions sperm mL^{-1}. The diluted semen was cooled to + 4°C in the refrigeration showcase for one hour and 20 min. During this period, we prepared the straws

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(0.5 mL) which had been already identified in the refrigerated display case. Finally, the straws were automatically filled at + 4°C. The freezing of straws was performed using a programmable freezer which provided the decrease of temperature from+4-140°C at the velocity of-20-60°C min^{-1} during 2 min and 30 sec. At the end of the freezing, the straws were stored in a tank of liquid nitrogen at-196°C.

Sperm morphology features: Two straws per ejaculate were thawed in a water bath at 37°C for 30 sec. The semen straw (0.5 mL) were diluted in 2 mL of INRA 96® which was previously placed in a water bath at 37°C and then incubated for 10 min at the same temperature. Sperm morphology was determined using eosin-nigrosin stain. From each thawed straw, two droplets of stain and semen (2 μL each one) was mixed and smeared on a prewarmed glass slide (37°C). The slide was examined under light microscope at ×400 magnification (Brito, 2007). At least 150 sperm were examined and classified into 4 different classes according to their features: percentages of abnormal head, abnormal mid piece, abnormal flagella and percentage of sperm with droplets. After that, the percentage of total abnormal sperm was estimated.

Statistical analysis: Data were analyzed by Statistical Analysis System (SAS Institute Inc., Cary, NC, USA). General differences between means were tested using factorial analysis for unbalanced data (GLM procedure of SAS). We took into consideration the effect of stallions, ejaculates, age, years of the freezing. The comparison of sperm morphology parameters between variables cited above was carried out using the DUNCAN test. Significance was considered at $p<0.05$.

RESULTS

Our results showed that all sperm abnormalities varied both between stallions and between ejaculates within stallion ($p = 0.0001$). A significant effect of stallion's age on the percentage of abnormal flagella ($p = 0.0001$) and total abnormal sperm ($p = 0.0397$) was observed (Fig. 1).

We also showed that the percentage of total abnormal sperm varied within freezing year, and it was about 34±2.2 and 37±1.8 %, respectively for 2009 and 2010 ($p = 0.0518$; Table 1).

DISCUSSION

Our results showed that all abnormal sperm, except proximal and distal droplets after semen thawing, were higher in young stallions than in old ones. Previous study in our laboratory (Najjar *et al*., 2010), using fresh semen of the same stallions, showed that abnormal sperm was higher in old stallions than the young ones (31.4 vs. 25.7%). Thus, and according to these findings, it seems that young stallion's sperm support less the freezing process when compared to the old ones. Besides, and always according to our previously findings on fresh semen, we noted that the freezing process increased the percentage of total abnormal sperm in both classes of age. The increase of sperm abnormalities could be attributed to cryopreservation. In fact, it was shown that crypreservation may generate morphological alterations (Bailey *et al*., 2000) and membrane destabilization (Holt and North, 1986) of mammalian sperm. Christensen *et al*. (1995) observed ultrastructural changes in the acrosome, the outer fibres of the midpiece, and the axoneme of the principal piece after freezing and thawing processes. Besides, Varner (2008) reported that cytoplasmic droplets seems to have a minor effect on fertility of stallions. However, detached head, abnormal mid pieces and premature germ cell had noxious effect on fertility.

The important variations between stallions and between ejaculates within stallions were shown by many others authors (Blach *et al*., 1989; Katila, 2001). In fact, Blach *et al*. (1989) reported that morphological sperm

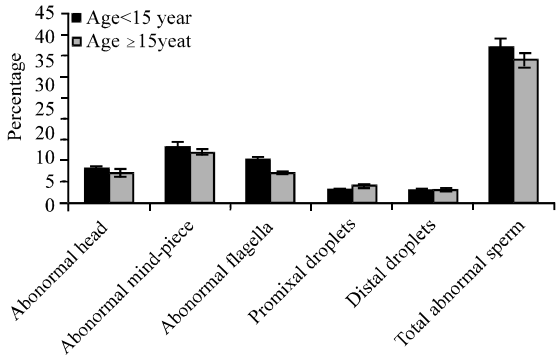

Fig . 1: Sperm morphology features in young and old stallions (Means±sem)

Table 1: Sperm morphology features according to years of thawing (Means±sem)

Year	Abnormal head (%)	Abnormal mid-piece (%)	Abnormal flagella (%)	Proximal droplets (%)	Distal droplets (%)	Total abnormal sperm (%)
2009	8±1.2	12±1.4	8±0.6	3±0.4	3±0.4	34±2.2[a]
2010	7.2±0.5	13±1.1	10±0.6	4±0.5	3±0.3	37±1.8[b]

[a, b]Significant at: $p = 0.0518$

variation is due to the freezing and thawing processes. Katila (2001) found that these variations were assigned to sperm ability to support the freezing and thawing processes.

CONCLUSION

It is clear that freezing and thawing processes increased sperm abnormalities that are due to cell alteration of young and old stallions. Consequently, we can say that abnormal spermatozoa affects the quality of the thawed semen of Tunisian Arab stallions.

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