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EFFECTS OF TIME STORAGE, EGG YOLK CONCENTRATION AND SEMEN WASHING ON SPERM MOTILITY IN ARBIA BUCKS

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ABSTRACT

With a prospect to improve goat artificial insemination, this study aims to evaluate the effects of storage time, egg yolk concentration and semen washing on the rate of motile spermatozoa (spz) in Arbia breed bucks semen stored at 5 °C for 48 h. Semen was collected from three mature bucks using an artificial vagina during December 2018. The ejaculates used in this study (rate of motile sperm > 75 %) were divided into two fractions: A, and B, (unwashed and washed samples, respectively): themselves divided into three fractions, and diluted in Tris-Acid citric based extenders, containing egg yolk at 2 %, 10 % and 20 %. ANOVA test was performed using the R software version 3.3.0. The rate of motile spermatozoa was evaluated after 24 and 48 hours of storage in the refrigerator at 5 °C. It registered a significant decrease as a function of the storage time in the different extenders. It varied between 42.43 ± 9.86 % and 61.29 ± 5.44 %, after 24 hours of storage, and between 35.86 ± 13.95 %, and 55.45 ± 11.65 % after 48 hours of storage. The semen washing significantly reduced the rate of motile spermatozoa in the different extenders. Increasing the concentration of egg yolk in the extender increased the rate of motile spermatozoa, especially during the first 24 hours of storage. In conclusion, the rate of spermatozoa motility of unwashed semen stored at 5 °C is higher in extenders containing high egg-yolk concentration, and decrease according to conservation time.

Key words: Arbia bucks; conservation; egg yolk; semen washing; sperm motility

INTRODUCTION

Semen cryopreservation, when associated with artificial insemination, represents an efficient mechanism to the promotion and diffusion of genetic material [19]. Storing semen in a liquid state can be an alternative to the semen freezing – thawing for artificial insemination [29]. During the process, spermatozoa are subjected to low temperature which may alter their viability, structural integrity, motility and consequently conception rates [4].

Many extenders have been produced, some empirically, to protect and maintain sperm fertility during semen examination and storage [37]. Egg yolk is an important ingredient of Tris based semen extender [18].

The problem with the semen goat extenders containing egg yolk is due to the presence of egg yolk coagulating enzyme (EYCE) in seminal plasma, which can be harmful to the sperm cells [43].

Many researchers recommend different egg yolk concentration in extender for various goat breeds, giving an impression of breed specific variation in respect to egg yolk concentration [4].

Semen washing is the conventional method of overcoming these harmful interactions, while some reports found positive results for sperm frozen without washing [45].

Therefore, the aim of the present study was to evaluate the effects of time of semen preservation at 5 °C, egg yolk concentration and semen washing on the rate of sperm motility in Algerian Arbia breed bucks.

MATERIALS AND METHODS

This work is part of a doctoral thesis defended at the Blida Institute of Veterinary Sciences under number 24/ISV/2019.

Location and experimental period

The study was carried out at the experimental Animal Farm of University of Tiaret during December 2018. Tiaret city is situated in western Algeria at latitude of 35°15' N and longitude of 1°26' E [2]. Climatologically, this region is a semi-arid area characterized by cold and humid winter and hot and dry summer [1]. The daily photoperiod varies between 9 h 37 min during the winter solstice and 14 h 23 min during the summer solstice.

Animals

Three healthy mature Arbia bucks 04 years old were used in the study. The bucks were kept in individual stalls and subject to a natural lighting. They receive a daily diet of 500 g barley, and have free access to straw and water. A vitamins and minerals supplements were incorporated in the ration, in the form of a salt lick.

Semen collection and evaluation

Semen samples were collected from each buck once a week using an artificial vagina. Immediately after collection, the ejaculates were placed in a water bath (37 °C) and transferred to the laboratory. Once in the latter, the volume of the ejaculate, sperm concentration and sperm motility were assessed. The ejaculates chosen to undergo the conservation at 5 °C were those whose initial individual mobility was greater than 75 %.

Each ejaculate was split into two equal parts: A and B in tubes, kept at a temperature of 28 °C [35].

Part A of the ejaculate was divided into three equal volumes without semen washing. For part B of ejaculates, a washing solution based on Tris – citric acid – fructose was added to the tube and centrifuged at 600gr/min for 15 minutes at a temperature of 28 °C [11]. The volume added of this washing solution varied according to the semen sample volume, it was five times the volume of the latter. After centrifugation, the supernatant was pipetted out with gentle aspiration; the sperm pellet that sedimented in the bottom of the tube was re-suspended carefully by adding Tris – citric acid – fructose solution to obtain the initial ejaculate part B volume and divided into three equal volumes.

A Tris – citric acid – fructose based extender containing different concentrations of yolk (2, 10 and 20 %) was added to the samples obtained from parts A and B of the ejaculates at a temperature of 28 °C, to obtain a concentration of 4×10^6 spermatozoa per straw of 0.25 ml. So, the required volume of extender depends on the initial volume and concentration of the ejaculate [7, 35].

The tubes containing the samples obtained after diluting part A and part B of the ejaculate were placed in a beaker containing about 200 ml of water at room temperature and placed in the refrigerator at 5 °C. Two hours later, the identified artificial insemination straws were filled by oral aspiration and sealed with polyvinyl alcohol powder [35].

The rate of motile spermatozoa was assessed after 24 and 48 hours of storage. Straws were warmed by plunging them in a water bath at 37 °C for 30 sec [19]. Individual sperm motility evaluation was performed by diluting the semen in normal saline and estimating the number of motile and non-motile spermatozoa. This assessment was carried out by focusing the binocular microscope at the centre of the cover slip at 40 × magnification (B – 350, Optika

Microscopes, Italy). Slide and cover slip temperature was maintained at 37 °C by using a heating stage fitted to the microscope [7, 30].

Statistical data analysis

Data were analysed using analysis of variation procedure (ANOVA), and the HSD Tukey test of the R software version 3.3.0 (2016–05–03). The statistical study concerned the influence of washing (washed or not), egg yolk concentration (2, 10 and 20 %) and period of preservation (24 and 48 h) on the rate of spermatozoa motility. The level of significance of statistical analyses was assessed based on the p-value. The difference between the variables was significant if the P-value < 0.05.

RESULTS

The means of volume of the ejaculate, sperm concentration and sperm motility in fresh semen were 1.18 ± 0.4 ml, 5.32 ± 2.82 spz.ml^{-1} and 79.08 ± 7.83 %, respectively. However, in relation to the volume and concentration of ejaculates, the number of straws obtained varied between 4 and 14 straws per ejaculate, with an average of approximately 9 straws per ejaculate.

The results of the semen storage at 5 °C in the extender based on Tris – citric acid – fructose with different concentrations of the egg yolk are summarized in Table 1 and Figures 1 and 2.

Table 1. The sperm motility rate of Arbia breed bucks according to storage time, semen washing and egg yolk concentration

Time of conservation	Semen washing	Level of egg yolk (%)	Mean of motility* (%)
24 hour	Washing	2	42.43 ± 9.86
		10	52.14 ± 13.01
		20	55.14 ± 12.59
	Without washing	2	55.14 ± 38.14
		10	58.71 ± 6.60
		20	61.29 ± 5.44
48 hour	Washing	2	35.86 ± 13.95
		10	39.57 ± 13.10
		20	38.57 ± 15.73
	Without washing	2	55.45 ± 11.65
		10	45.71 ± 16.20
		20	44.71 ± 19.70

* Data are expressed as mean ± standard deviation

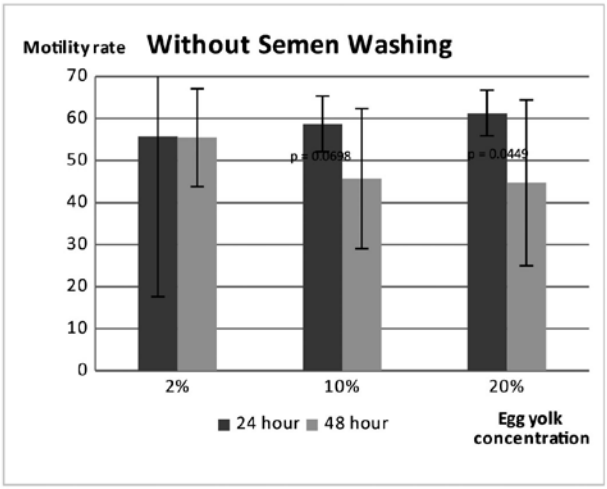

Fig. 1. Rate of sperm motility without semen washing

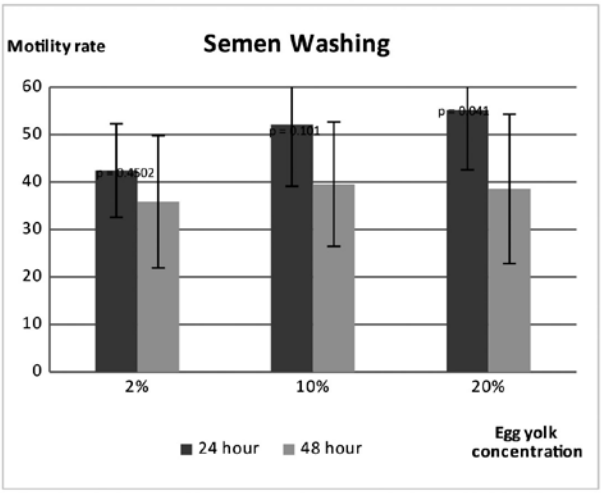

Fig. 2. Rate of sperm motility after semen washing

Statistical analyses of our results show that storage time and semen washing have a significant influence (P < 0.05) on the rate of motile spermatozoa. On the contrary, the concentration of the egg yolk in the extender showed no significant influence (P > 0.05) on this one (Table 2).

Table 2. Results of statistical analyses of factors (time of storage, egg yolk concentration and washing) influencing the sperm motility rate

Factor	P value
Time	2.71e^{-12} ***
Concentration	0.12059
Washing	0.00423 **
Time × Concentration	0.63041
Time × Washing	0.18286
Concentration × Washing	0.65136
Time × Concentration × Washing	0.19866

The asterisks indicate that the factor influences significantly the motility rate (P < 0.05)

Table 3. Results of statistical analyses of time of preservation effect on sperm motility

Time of preservation			0 h vs 24 h	0 h vs 48 h	24 h vs 48 h
P-value	Without washing	2 %	0.0000586	0.0000000	0.0011960
		10 %	0.0071716	0.0000428	0.0698758
		20 %	0.0470145	0.0001745	0.0449525
	Washing	2 %	0.0000094	0.0000009	0.4502721
		10 %	0.0008713	0.0000092	0.1010899
		20 %	0.0055171	0.0000196	0.0416732

Time of preservation

The average rate of motile spermatozoa in ejaculates in the different extenders decreases significantly as a function of time (Table 3). After 24 hours of preservation at 5 °C, it varies between 42.43 ± 9.86 % and 61.29 ± 5.44 % and decreases even more after 48 hours to 35.86 ± 13.95 % and 55.45 ± 11.65 %.

Semen washing

With regard to the washing of the semen, the average percentage of motile spermatozoa is significantly higher in the unwashed ejaculates than in those having undergone washing prior to their dilution (Table 4). This observation is valid for all extenders at different egg yolk concentrations during the first 24 hours of storage.

Table 4. Results of statistical analyses of semen washing effect on sperm motility

Semen washing		Without washing vs Washing
P-value	24 h	5.25e-05
	48 h	0.000702

Egg yolk concentration

Our results show that the rate of motile spermatozoa increases with increasing concentration of egg yolk in the extender, but without any significant influence on this one (Table 5). On the contrary, the rate of motile spermatozoa decreases with the increase in the concentration of the egg yolk in the extenders only when the semen is diluted as is after 48 hours of storage.

DISCUSSION

Time of preservation

In different goat breeds, several authors report, similar to our results, that the semen storage time has a negative influence on sperm mobility. C o r t e e l et al. [15] observed a drastic decrease in sperm motility when sperm is stored at low temperature at any time during the non-breeding season of Alpine and Saanen bucks. In the Murciano-Granadina [42] and Lubei [29] bucks, sperm mobility and viability, as well as acrosome integrity decrease with storage time. N g o u l a et al. [33] and P a u l e n z et al. [37] report similar findings to our results in West African Dwarf and Norwegian bucks, respectively.

Examination time point was identified by H a h n et al. [20] as factor influencing semen motility during fresh peacock goat semen assessment. C e t i n et al. [13] found that sperm motility of Damascus buck decreased during liquid storage at 4 °C according to preservation time in different extenders containing Tris-egg yolk or Tris-soybean lecithin supplemented with 0.25 %, 0.50 %, 0.75 % or 1 % concentrations of royal jelly.

The drastic decline in sperm motility of stored liquid semen may be explained by the gradual depletion of nutrients required for high metabolic demands such as potassium, sodium and plasma protein of sperm transport through the female genital tract [36].

Table 5. Results of statistical analyses of egg yolk concentration effect on sperm motility

Egg yolk concentration			2 % vs 10 %	2 % vs 20 %	10 % vs 20 %
P-value	Without washing	24 h	0.474	0.129	0.673
		48 h	0.662	0.732	0.992
	Washing	24 h	0.302	0.141	0.885
		48 h	0.474	0.129	0.673

Semen washing

Removal of seminal plasma is a process that can be time-consuming and can damage cells if performed incorrectly. However, if done correctly, removal of seminal plasma may be beneficial [40]. Then, the positive effect of semen washing may depend on the season of the year and the extender used [11].

In our study, washing semen does not appear to be a determining factor in improving sperm mobility as the latter is higher in unwashed semen samples than washed ones. This can be explained by the period of the study which corresponds, in terms of reproductive activity, to the end of a breeding season and the beginning of a transition period to a non-breeding season, knowing that the sexual activity of the Arbia bucks shows seasonal variations [3, 21]. On the other hand, seminal plasma contains various factors that interfere with the maintenance of the viability of spermatozoa and modulate their function [26]. Some proteins, correlated with semen quality parameters, showed significant seasonal variability [12]. As the molecular composition of seminal plasma is very complex and variable between species and males, the structure of seminal proteins and their specific effects on sperm may also vary [31]. So, all these observations comfort our work.

Nethenzheni et al. [32] concluded that there is a reduction in the percentage of progressive motility rate in unwashed and washed semen of indigenous bucks extended with Bioxcell® and Triladyl®. However, Bioxcell® extender was found to be more suitable for preserving spermatozoa during equilibration and freezing/thawing process of non-washed and washed buck semen.

Sperm washing removes the protective proteins and antioxidative enzymes [16]. Besides, semen supplementation with goat seminal plasma proteins obtained during the non-breeding season leads to deterioration in sperm quality [26], while those collected in the sexual season have a positive effect on sperm quality after freezing/thawing [27]. These authors' results can justify the results obtained in our study.

The secretions of the bulbo-urethral glands have a very negative effect on the survival of epididymal spermatozoa, whereas vesicular secretions have a positive effect on the mobility of the spermatozoa. Nunes et al. [34] suggest that the negative effect of bulb-urethral secretions on sperm motility is partially inhibited during the sexual season by large vesicular secretions, which is not the case in non-breeding season.

Our results are in agreement with those reported by Cabrera et al. [11] in Canary bucks. These authors show that the post-freezing survival of spermatozoa cryopreserved during the winter is significantly high when the semen was not washed. In Saanen bucks, Azeredo et al. [6] find that the average motility of spermatozoa collected between September and December and diluted in a medium containing egg yolk is high in sperm fractions containing seminal plasma, whether fresh or post-frozen.

Tuli and Holtz [44], Jimenez–Rabadan et al. [24], Ferreira et al. [19] and Peterson et al. [39] report findings similar to our results. Semen washing does not improve its quality after dilution and storage in extenders containing egg yolk (6.8 to 20 %) in bucks of Boer, Blanca-Celtibérica and Saanen breeds and bucks of artificial insemination centres, respectively.

Otherwise, some other research indicated that removal of seminal plasma is necessary to maximize post-freezing mobility and acrosomal integrity of goat sperm. Even though cryopreservation of Black Bengal bucks semen reduced sperm motility, semen washing had higher percentage of sperm motility compared to without sperm washing [16]. Semen washing improves its post-storage quality, in particular sperm motility in cross-breed bucks Beetal × Assam Locale [23], Saanen [45] and Alpine [25, 45].

Egg yolk concentration

Egg yolk is generally incorporated in semen extenders at different concentrations. However, it was also used at 20 % [10]. The harmful interactions between seminal plasma and egg yolks were first documented by Roy [43]. Regardless of their mechanism of action, activation of Egg Yolk Coagulating Enzyme in the extender is harmful to sperm quality during cooling and cryopreservation [38].

In this study, the egg yolk concentration did not appear to play a statistically significant role in the rate of sperm motility. It should be noted that the low concentration of the egg yolk (2 %) in the extender seems insufficient for maintaining good sperm motility, especially after removing of seminal plasma. On the contrary, the rate of motile spermatozoa increases with the increase in the concentration of the egg yolk in the extenders, especially when the semen is diluted as is without prior washing. These results can be explained by the breed susceptibility and the period during which our study was carried out, in this case the season of production of good sperm quality according to

B e l h a m i t i et al. [8, 9]. In addition, the effect of interactions between the enzymes of the seminal plasma and the egg yolk does not seem immediate, but rather cumulative as the rate of motile sperm increases and decreases with the increase in the concentration of the egg yolk in extenders during 24 and 48 hours of storage, respectively.

Levels of phospholipase A are lower in seminal plasma from semen collected during the breeding season compared to that collected outside the breeding season [19]. According to N u n e s [34], during the non-breeding season, the bulbourethral glands increase their activity by high plasma concentrations of prolactin and produce more phospholipase A. This enzyme catalyzes the hydrolysis of lecithin in egg yolk to fatty acids and lysophosphatidylcholine (LPC), which are toxic to spermatozoa [22, 43]. However, the lysophosphatidylcholine effects depend on the amount formed, temperature (enzyme activity and thus LPC formation is temperature dependent), dilution or degree of removal of seminal plasma, season of semen production and breed of fowl providing the egg yolk [28].

The addition of egg yolk to the semen extender significantly increases the sperm mobility of the white Lubei goat [29]. Also, the incorporation of 20 % egg yolk in the extender confers better cryoprotection to Barbari buck spermatozoa compared to 3 % egg yolk or 20 % egg yolk after washing [4]. D a s k i n and T e k i n [17] reported that during the breeding season, post-thaw sperm motility was better in extenders containing 20 % egg yolk than those without egg yolk. C h a u h a n and A n a n d [14] in Jamunapari bucks and A z a w i et al. [5] in Shami bucks showed that unwashed spermatozoa can be usefully preserved at low temperature after dilution in Tris-based extender with a high concentration of egg yolk. Our findings are in agreement with these results.

In contrast with our results, concentration of egg yolk higher than 12 % in extenders depressed post-thaw sperm motility and morphological integrity in Saanen bucks [45]. R i t a r and S a l a m o n [41] indicated that the effect of toxic products during incubation became apparent as egg yolk concentration increased but at a concentration of 1.5 % it does not exert a depressant effect on the viability of unwashed ejaculates in the Angora breed goat. Likewise, unwashed Murciano-Granadina spermatozoa can be usefully preserved at 5 °C after dilution in Tris-based extender with 2 % egg yolk [42].

CONCLUSIONS

Semen from Arbia bucks, collected in late autumn and early winter, can tolerate high concentrations of egg yolk (10 à 20 %) in Tris – citric acid – egg yolk based extenders to maintain sperm motility for 24 to 48 hours at 5 °C. Removal of seminal plasma by sperm washing does not appear to be essential to improve the liquid storage of semen in Arbia bucks. Therefore, further studies should be carried out to investigate the preservation of the semen during other times of the year and the *in vivo* fertility of this stored semen using artificial insemination.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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