CRYOPRESERVATION OF RAM SPERM FROM AUTOCHTHONOUS BREEDS DURING A NON-MATING SEASON

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ABSTRACT

It is possible to collect and successfully cryopreserve ejaculates in a non-mating season from rams of the autochthonous breeds Karakachan, Cooper-red Shumen and Karnobat-local, raised in Bulgaria. Studies are in progress aiming the elaboration of optimal cryoprotective extenders and freezing technology.

KEY WORD: Autochthonous rams breeds, sperm, cryopreservation

РЕЗЮМЕ

Възможно е да се получат и успешно криоконсервират еякулати от автохтонни кочове от породите Каракачанска, Медночервена Шуменска и Местна Карнобатска, в извънслучен сезон. Изследванията продължават, с цел оптимизиране на криопротективни среди и усъвършенстване на технологията за замразяване.

ПОДРОБНО РЕЗЮМЕ

Проведени са опити за криоконсервация на сперма от автохтонни породи кочове – Каракачанска, Медночервена Шуменска и Местна Карнобатска, отглеждани в България. Установено е, че криопротективната среда Triladil (Minitüb) запазва мотилитета на значителен процент от сперматозоидите след размразяване – обща подвижност на сперматозоидите (ОПС) 43.8% и сперматозоиди с праволинейни настъпателни движения (СПНД) 24.1%. Също така, преживяемостта на сперматозоидите при 39°C, изследвана до 300-та минута е съответно 7.4% за ОПС и 4.7% за СПНД. Структурните промени в сперматозоидите след размразяване са локализирани предимно в областта на флагелума и в по-малка степен в акрозомата. Резултатите от проведените изследвания дават основание да се приеме, че е възможна криоконсервация на сперма от автохтонни породи, получена в извънслучен сезон и съхраняване на генетичен материал в генетични банки.

INTRODUCTION

The genealogical descents of a great part of the sheep in the Balkan peninsula have common ancestors. Historically some of the present representatives of this animal species have been conserved and become differentiated in different regions as breeds. This has been conditioned by ecological factors in the Balkan countries which are favorable for raising different sheep breeds.

A small number of local autochthonous sheep breeds still exist in a state of good preservation in different regions of Bulgaria. In the past centuries a unique genetic stock of more than 15 native breeds has existed in the country. Recently their populations have strongly decreased which calls for new approaches to preserve the genetic fund. This might be achieved by long-term use of valuable rams from autochthonous breeds as sperm donors during the non-breeding seasons to create a sperm gene bank. It will enable the optimal use of the brood rams and insemination of a large number of ewes, hence preservation of the breeds.

The problems of sperm cryopreservation occupy an important place in modern biology of reproduction. Scientific investigations have resulted in new possibilities to preserve the viability and fecundity of male gametes at ultralow temperatures. Consequently the methods of cryopresevation enable us to keep in a gene bank frozen sperm of native species and breeds for artificial insemination during unlimited periods of time.

Data in the literature indicate great theoretical and practical interests in the ovine species [6, 8, 9, 11, 13, 14]. It has been established that ram spermatozoa are sensitive to the conditions of low-temperature anabiosis and freezing [9]. Moreover there are some differences in the cryotolerance of the gametes and the causal relationship with the fecundity [11]. Some authors take a great interest in the role of cryoprotectants and technologies for cryopreservation and artificial insemination [8,1,2, 14], as well as in the two-stage dilution to prevent the toxicity of glycerol [4,5]. The basic criterions for evaluation of the sperm quality (motility, survival and structural changes of spermatozoa) give information about the biological potentialities of the male gametes [8,9,4,12]. Fair results have been attained by laparoscopic insemination with ram semen frozen in straws (40-60% fertility rate) [2]. Long-term storage of sperm from rams of valuable breeds is economically efficient when collection and freezing of ejaculates are performed all the year round [8]. We have initiated studies on the possibilities for cryopreservation of sperm collected from rams of autochthonous breeds in all seasons. It is possible to induce sexual reflexes and to collect sperm from rams of Bulgarian native breeds in non-mating season [10]. No attempts however have been made so far to freeze sperm from autochthonous rams. It is the aim of the present study to collect and cryopreserve semen from rams of local autochthonous breeds in a non-mating season in order to create a gene sperm bank.

MATERIALS AND METHODS

Rams of autochthonous breeds raised in different regions of the country were brought together in a shelter of the Agency for Selection and Reproduction in Sofia. After a 45-days period of adaptation a total of 36 ejaculates were collected by the artificial vagina method from 3 rams of the Karakachan breed, 1 ram of the Cooper-red Shumen breed and 1 ram of the Karnobat-local breed in a non-mating season (April-May).

Sperm quality was assessed by determination of the volume of the ejaculates (cm^3), concentration of spermatozoa (10 /cm^3), sperm cell motility (%), normal and pathological

spermatozoa (%) and pH of sperm [15]. The total sperm motility was estimated by dividing the live and dead gametes after staining with 0.1% eosin and 1% of $KMnO_4$ [8]. Cell counting was performed in a Burker's chamber. The suitability of the ejaculates for cryopreservation was evaluated and prognosticated by determination of the temperature resistance of spermatozoa. The fresh sperm was incubated in a water bath at 35°C for 10 min. Then semen samples of 0.2 cm^3 were transferred to two test tubes by a micropipette. The first test tube was kept in a water bath at 37°C for 3 min and the second in an ice bath

Table 1. Biological characteristics of ram sperm

Breeds	Ejaculates studied (n)	Volume (cm^3)	Motility (%)	Concentration ($10^9/cm^3$)	Pathological spermatozoa (%)	pH
Karakachan	20	1.46±0.05	83.50±3.66	2.46±0.07	17.40±1.42	6.72±0.03
Cooper-red Shumen	8	1.05±0.02	81.30±2.31	2.51±0.08	18.80±1.59	6.71±0.03
Karnobat-local	8	1.14±0.01	82.50±4.60	2.64±0.06	16.20±1.63	6.74±0.04
Total	36	1.29±0.06	82.80±3.29	2.51±0.07	17.43±1.39	6.72±0.04

Table 2. Resistance of spermatozoa to temperature shock

Coefficient for cold resistance of spermatozoa	Number of ejaculates	n ± Sx
> 0.30	33	0.39± 0.03
< 0.30	3	0.17±0.02

at 0°C for 3 min. The percentage of motile spermatozoa was determined microscopically [11].

Ejaculates evaluated as suitable were frozen by the Cassou method in straws of 0.2 cm^3 in the extender Triladil (Minitüb) containing 25% egg yolk. Sperm was diluted to a final dilution of 120 millions spermatozoa in a dose. The samples were cooled at 2-5°C for 4 hours and then held on liquid nitrogen (LN_2) vapors for 9 min. Finally the straws were transferred to a LN_2 container for storage at -196°C. Straws were thawed in a water bath at 39°C after storage in LN_2 for 24 hours. Thawed sperm samples were evaluated according to the following parameters: total sperm motility (TSM) (%), spermatozoa with straight-forward movement (SSFM) (%), temperature resistance at 39°C for 300 min (%). Data were processed under the Student's variation-statistical method.

RESULTS AND DISCUSSION

The estimation of the biological indices of the collected ejaculates (n=36) indicated that 33 of them are appropriate to cryoprotection. Data of the spermatological investigations prior to cryoprotection are given in Table 1.

The results of the studies on the resistance of spermatozoa to temperature shock are given in Table 2.

Results demonstrate that the sperm cells in 33 (92.3%) out of the 36 ejaculates have a high cryoresistance (>0.30) and preserve their viability during severe decrease of the temperature from 39°C to 0°C. The mean value of the resistance coefficient of spermatozoa to temperature shock is 0.39±0.03 which enables their cryopreservation. Data also clearly indicate that semen from autochthonous ram breeds frozen in straws by us, after thawing preserve the spermatozoa motility to a considerable extent. Comparatively high values were obtained of the index of the total motility (%) and a little lower values of the percentage of spermatozoa with SSFM (Fig.1).

The alive spermatozoa were not stained while the dead cells were pink stained. The extender Triladil exerts a good cryoprotective effect preserving to a great extent the sperm motility after thawing the ejaculates. Thawed spermatozoa also have a good thermoresistance regarding to both TSM and SSFM. Survival of spermatozoa at 39°C followed to 300 min, is 7.4% and 4.7% for TSM and SSFM, respectively.

Morphological characteristics of the thawed spermatozoa were made to determinate of cryotolerance because all the ejaculates were collected and frozen in a non-mating season. The results are shown in Fig.2.

The morphological studies revealed that freezing and thawing of the ejaculates affect a portion of the gametes

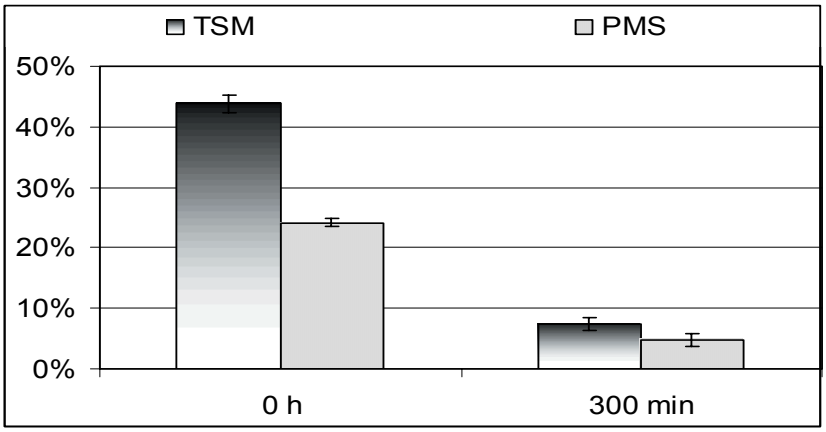
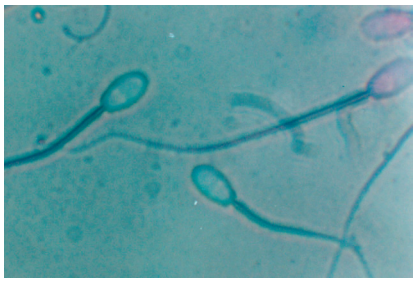
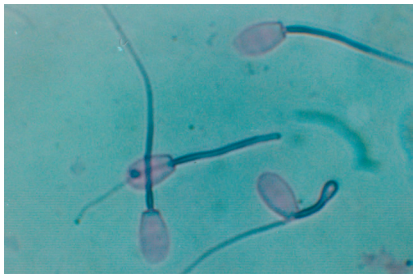


Fig.1. Motility and survival of ram spermatozoa after cryopreservation at -196°C and thawing at 39°C



a



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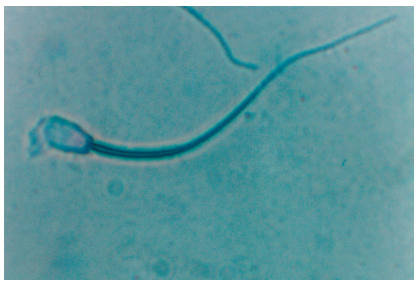

c

Fig.2. Morphologic appearance of live and dead spermatozoa – a. live and dead spermatozoa; b. changes in tail and mid piece; c. sperm cells with changed acrosome.

and as a result structural abnormalities appear. These anomalies are located predominately in the flagellum and more rarely in the acrosome. It is most likely that the cryogenic damages are due to irreversible destruction in single components of the structural organization of sperm cells. Both osmotic and temperature gradients cause changes in the molecular organization and function of the plasma membranes and the membranes of the intracellular organelles at low temperature influences on spermatozoa [1, 7, 12]. On the other hand a portion of the ejaculates collected during a non-mating season have lower values of the index for TSM and a higher percentage of abnormal spermatozoa prior to freezing. The cryoresistance of those gametes is decreased.

On the grounds of the experimental results it might be assumed that ram sperm from autochthonous breeds collected in non-mating seasons can be stored as a genetic material.

The temperature resistance of the gametes in the ejaculates is fair and they can be cryopreserved. During the procedures of freezing and thawing the motility, viability and morphologic structures of spermatozoa are preserved to an acceptable extent. Presumably such ejaculates can be collected and stored in sperm cryobanks for future use. The encouraging preliminary results inspire us to continue and extend the research studies on collection of normozoospermic ram ejaculates in non-breeding seasons, cryopreservation, sperm banking and conservation of the endangered autochthonous sheep breeds.

CONCLUSION

Our study on ejaculates collected and frozen in a non-mating season from rams of the autochthonous Karakachan, Cooper-red Shumen and Karnobat-local breeds indicated that it is possible sperm doses to be successfully cryopreserved in straws. The cryoprotective

extender Triladil protects 43,8% of the total sperm cell motility and 24.1% of the spermatozoa with straight forward movement after thawing. Structural changes affect mainly the flagellum and to a lesser extent the acrosome.

Conservation of autochthonous sheep breeds is possible also by artificial insemination with cryopreserved semen collected in a non-mating season.

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