ROLE OF TREHALOSE IN THE PROTECTION OF THE REFRIGERATED BUCK SEMEN

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Abstract

The biotechnologies of reproduction, that also involves the artificial insemination at goats, have been rapidly developed after the optimization of producing and preserving the semen methods and of those of synchronizing the females` estrum. In order to obtain an acceptable fertility is necessary that optimum methods of preserving the semen in refrigerated form to be developed, which can be easily and fast integrated into insemination methodologies. The aim of the study was to test a new medium of dilution which contains a diglucide trehalose as cryoprotectant substance, for the purpose of replacing (to replace) the usual glycerol that has been used until present, which was proved to have negative effects upon spermatic cell during preservation. Also the extender contains a low concentration of egg yolk of 5%, in order to eliminate a processing-centrifugation step, which leads to an additional mechanical stress with negative effects on fecundity.

Our experiments demonstrated that the trehalose added in the dilution medium maintains the structural and functional stability of the plasmatic membrane of the spermatic cell during liquid conservation at 4 degrees C, similar results being obtained also in other experiments done on buck semen. At 48 hours from collection, the semen diluted in the control extender registered a motility of 64.1% while the semen diluted in the experimental extender registered a motility of 67.4%. At 48 hours from collection, the semen in the control extender registered a viability of 66.4% while the semen in the experimental extender registered a viability of 69.5%.

Key words: seminal material, buck, trehalose extender

INTRODUCTION

The techniques of assisted reproduction, especially artificial insemination, were used successfully to optimize the efficiency of reproduction at animals during the last years.

At goats, the biotechnologies of reproduction, which imply also the artificial insemination, have developed rapidly after the optimization of producing and preserving the semen methods and of those of synchronizing the females` estrum. In order to obtain an acceptable fertility is necessary to develop optimal methods of preserving the semen, in refrigerated or freeze form, that can be easily and rapidly integrated in the insemination methodologies.

The composition of extender is critical for the success of the liquid preservation at low temperature of the buck spermatozoa. The extenders used for semen dilution should give an optimum buffering capacity and an energy source as metabolising sublayers that give to motility the necessary energy and the spermatozoa can use them both in aerobe and also anaerobe conditions. Also, they should offer protection against bacterial infection and osmotic and thermic stress. The role of seminal plasma upon the spermatozoa was studied on a large scale and there were obtained various results regarding the activation of motility, keeping the osmotic pressure and the source of nutrients, prevention against the early activation during female insemination and stabilization of cellular membrane by capacity inhibitors and the influence upon fertility [20]. The seminal plasma contains proteins which are epididymal gland secretions and secondary sexual glands. Secretion and excretion of certain proteins during semen maturation in the epididymal glands and when the semen is ejaculated, have an important role in keeping the stability of cellular membrane and motility. Some proteins from the seminal plasma prevent damaging the spermatozoa by thermic

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The manuscript was received: 24.08.2018
Accepted for publication: 14.09.2018

shock [14]. The types and quantity of seminal plasma proteins may vary depending on individuals. These proteins can be affected by certain environment factors as the season when the semen is collected, stress, food, temperature [13] or even the seamen collecting method [11].

Other compounds of the seminal plasma are the enzymes. An enzyme which is believed that play a role during acrosome reaction and spermatozoa-oocyte fusion is the A2 phospholipase. At buck, coagulating egg yolk enzyme (EYCE) and the SBUIII enzyme (bulbourethral III secretion) are secreted by the bulbourethral glands and have phospholipase activity. Coagulating egg yolk enzyme (EYCE) hydrolyzes the lecithin from the yolk extender in fatty acids and lysolecithin, which is toxic to spermatozoa [18]. This hydrolysis induces acrosome reaction and chromatin decondensation [19]. As a result of these reactions is a decrease in the percentage of motile spermatozoa, deterioration in the quality of movement, breakage of acrosomes and finally, cellular death [10]. The toxicity of these enzymes differs with pH, temperature, seminal plasma concentration and season of semen production [10].

In order to avoid the harmful effects of these enzymes on egg yolk and skim milk, some authors have suggested that the removal of seminal plasma from ejaculates by means of centrifugation is an useful method to increase the sperm motility, membrane integrity and fertility after freezing-thawing procedure [9]. Other studies showed that removing the seminal plasma does not have any effect on semen quality after thawing [5].

The objective of the study was to test a new extender which contains a dyglucide-trehalose as cryoprotectant substance, with the aim to substitute the usual glycerol used until now, that was demonstrated to have a harmful effect on sperm cell during preservation. Also the extender contains a low concentration of egg yolk of 5%, in order to eliminate a processing stage - centrifugation, which leads to a supplementary mechanical stress with negative effects on fecundity.

MATERIALS AND METHODS

Location and animals

The experiments of collecting, diluting and conserving through refrigeration of the buck semen samples were done during the period September-January 2017. Preparation and physical and chemical analysis of the extender were obtained in the Laboratory of Biotechnologies of Reproduction at ICDCOC Palas Constanţa. The experiments of collecting and processing the semen were performed at the goat farm from ICDCOC Palas Constanţa.

The semen came from 10 buck of Carpathian breed with the age between 1.5 and 2 years.

Extender

The extender made from Tris- citric acid – glucose - yolk (20%)-glycerol 2% was used as control extender. The experimental extender contains Tris - citric acid - glucose-trehalose - yolk (5%).

Collecting the semen

For each male, the electroejaculator was used. The collection was performed during breeding season, once per week, followed by a break of 2 weeks. The collection of seminal material was done in collecting glasses maintained in water bath at 37^0C.

Before being evaluated in the laboratory, the semen is maintained on water bath at 37°C, right after being collected. The analysis of the semen was done in approximately 10 minutes from collection. The macroscopic evaluation of the semen includes: volume, motility, concentration and pH.

The volume of the semen collected by electroejaculation was gathered by reading the scale of the collecting glass. The semen was collected in graded Falcon tubes.

The semen's pH was measured right after collection with a Consort pH-meter. In order to read the pH, the electrode was washed with distilled water, dried with sterile filter paper and then introduced into the semen sample. After each semen sample, the electrode was sprayed with 70% ethanol solution.

The concentration is in terms of milliards of spermatozoa/ml semen. The semen accepted for dilution should contain 3,0-6,0 milliards spermatozoa per ml.

The motility is a very important sperm indicator on which the fecundate capacity depends. The motility evaluation is performed in order to find the ejaculated samples with dead sperm cells or with very small motility. The minimum accepted motility is 70% for raw semen and 80% for semen which will preserved by dilution.

The motility evaluation should consider the speed of spermatozoa moving, linearity index and amplitude of lateral head displacement. To evaluate the motility of the semen it is classified in non-motile, with progressive motility or with non-progressive motility. It is also evaluated the total percentage of the spermatozoa with progressive motility.

The motility was estimated by the evaluation technique in wet medium, at the optical microscope (Novex, Holland) (x100 zoom) with hot plate maintained at 37°C and camera [21].

Dilution and placing in the fridge for cooling (+4 - +5^{0}C) of the recipient with diluted semen on water bath.

After qualitative evaluation, the semen was mixed and divided in two parts: first part is processed by the classical method (centrifugation, dilution with control medium which contains egg yolk 20% and glycerol 2%), and the second part is diluted without centrifugation with an experimental extender which contains trehalose and a low concentration of egg yolk (5%).

RESULTS AND DISCUSSIONS

The dilution of the semen is performed with the aim of increasing the volume of an ejaculate, in order to inseminate a higher number of females. The development of the semen preserving technology proved that survival for longer periods of conserved spermatozoa is in opposite relation with their metabolic activity.

The extender composition is critical for the success of the buck spermatozoa liquid preservation at low temperature. The extenders used for semen dilution must provide an optimal buffering capacity and an energy source as metabolising sublayers that give to motility the necessary energy and which the spermatozoa can use them in aerobe and anaerobe conditions. Also, they should offer protection against bacterial infection and osmotic and thermic stress.

At the present moment, for semen preservation the most used extenders are those based on Tris and creamed milk. Except Tris, the extender also contains fructose or glucose, egg yolk, antibiotic and citric acid.

Our studies were focused on obtaining and testing a new, cheap and easy dilution medium, that allow buck semen dilution and preservation by refrigeration and lead to an increase of qualitative parameters of the semen after preservation.

After qualitative evaluation, the semen was mixed and divided into two parts: first part is processed by the classical method (centrifugation with control medium with 20% egg yolk), and the second part is diluted with an experimental medium, without separating the seminal plasma from the spermatic cells. The experimental medium contains trehalose and a low concentration of egg yolk (5%).

Both extenders used contain glucose (a monoglucide which is used by the sperm cell in order to obtain cellular energy) and also Tris (hydroxymethyl) aminomethan and citric acid for pH buffering.

The buck semen has a feature which must be considered in the dilution processes due to the interaction of seminal plasma with the egg yolk which can be harmful to the semen of this species. This happens because the semen has an enzyme secreted by the bulbourethral glands, which produces the hydrolyse of the lecithin from the egg yolk and lisolecithin which is toxic for the sperm cell. That is why an additional stage of removing the plasma by centrifugation is needed.

The same basic substances from Tris base extender that are usually used for the dilution of buck semen are also used in our studies but with a lower concentration of egg yolk (5%) and without glycerol, that was proved to be toxic in long preservation. The medium contains trehalose, which has also antioxidant activity.

Qualitative parameters variation of refrigerated semen

The semen collected for dilution was taken from 10 buck of Carpathian breed with the age between 1.5 and 2 years. The electoejaculator was used for collecting the

ejaculates from each male. The artificial vagina (AV) is usually used for the collection at small ruminants but when males are not trained for artificial vagina the electroejaculation (EE) is an alternative.

The collection was performed once a week, followed by a break of 2 weeks. In total there were collected 60 ejaculates.

The volume was evaluated right after semen collection and resulted an average of 1.2 ml per ejaculate (Table 1). The average is in normal values of 0.8 and 2.0 ml for buck semen. There are differences in the characteristics of the ejaculates obtained by different collecting methods and it was noted that electro-ejaculation is different from the physiological ejaculation. Thus the electro-ejaculation artificially stimulates accessory glands with electrical stimuli, causing a volume increase that means a higher quantity of seminal plasma.

The spermatic indicators for the raw semen that was gathered by electro-ejaculation from the buck of Carpathian breed are presented in table 1.

Table 1 The spermatic indicators for semen samples collected by electro-ejaculation (average ± standard deviation)

Breed	n	Number of Ejaculates	Vol ejac/ml x±Sx	M% x±Sx	Concentration $x10^9$spz/ml x±Sx	pH
Carpathian	10	60	1.2±0.3	86.1±5.1	4.15±0.65	6.9±0.12

Note: n= number of buck; M= motility of raw semen

The pH of the semen was measured using a pH-meter right after collection. The electrode was washed with distilled water and dried with sterile filter paper, then introduced into the semen sample to read the pH. The result was an average value for pH of 6.9. This pH is accepted because the buck semen pH varies between 7.0 and 7.8, but the pH of the semen obtained by electro-ejaculation method (6.9 ± 0.12) is more acid, a fact that is also confirmed by other studies on buck. The semen acidity is an indicator of excess secretion of accessory glands due to electrical stimulation in the rectal zone of the buck.

The excess of the accessory glands is also responsible for semen preservation capacity. The same researchers reported that spermatozoa motility is affected in an acid pH environment, maybe because of the changes in metabolic activity and a deregulation in the breath of sperm cells.

Motility variation of refrigerated semen

The semen motility was evaluated in both extenders right after collection. A semen motility of 81.3% was obtained in a control medium while a motility of 85.2% was in the experimental extender. Both samples were refrigerated at temperature of +4-+50C. The semen motility was evaluated for both extenders (Table 2) at every 24 hours. The semen motility was 75% in the control medium, while a motility of 80.3% was obtained in the experimental medium.

Table 2 The effect of extender upon motility (%) during the refrigeration period (4^0C)

Refrigeration period (h)	Control medium Tris+egg yolk 20%	Experimental medium
0	81.3±4.37	85.2±1.44
24	75.0±5.67	80.3±2.57
48	64.1±3.95	67.4±3.66
72	45.4±4.96	55.5±5.41
96	36.3±4.82	40.4±2.84
120	25.5±3.67	27.0±2.11

The semen, in the control medium, at 48 hours from collection had a motility of 64.1%, while the semen in the experimental medium had a motility of 67.4%. At 72 hours from collection the semen in the control medium had a motility of 45.4% while the semen in the experimental medium had a motility of 55.5%.

At 96 hours from collection the semen in the control medium had a motility of 36.3%, while the semen in the experimental medium had a motility of 40.4%. At 120 hours from collection the semen in the control medium had a motility of 25.5% while the semen in the experimental medium had a motility of 27%.

These results confirmed the studies of Azeredo [3] which reported harmful effects on semen motility after centrifugation and addition of the extender based on egg yolk 20%. The seminal plasma removal can be harmful for the semen by decreasing the presence of the antioxidants in seminal plasma. The absence of these substances could accelerate the action of oxygen reactive types which decrease the motility, viability and integrity of semen DNA.

On the other hand, the results in this study are not similar to those observed by Memon [12], Ritar and Salamon [15] and Sariozkan [17], that reported benefits upon the quality of the buck semen by centrifugation and removal of the seminal plasma. The positive results can be explained by the fact that samples were collected and diluted at the end of the breeding season, when the concentration of A phospholipases from the seminal plasma is smaller than that collected during the breeding season. Also it is possible that in this work, the results to have been influenced by age, as young bucks were used (1.5-2 years). The young animals have the reproductive system still in developing progress and the level of producing the A phospholipases is lower comparing to adult animals, that have already well developed the bulbourethral glands.

Determination of viability is one of the basic elements of evaluating the quality of semen, with a great importance in the distinction between dead and alive immotile spermatozoa, especially for the samples with many immotile spermatozoa, To appreciate the viability it was used the eozine-nigrozine colouring method.

Viability variation of refrigerated semen

A semen viability of 85.2% was obtained in the control medium while a viability of 86.6% was resulted for the semen in experimental medium right after collection (Table 3).

Table 3 The effect of extender upon structural integrity of the membrane (viability, %) per refrigeration period (4°C)

Refrigeration period (h)	Control medium Tris+egg yolk 20%	Experimental medium
0	85.2±4.36	86.6±1.44
24	77.1±5.67	81.2±2.57
48	66.4±3.93	69.5±3.68
72	47.5±4.98	56.4±5.41
96	37.6±4.84	42.3±2.85
120	24.3±3.65	29.1±2.12

Both samples were refrigerated at the temperature of +4-+5°C. At every 24 hours the semen viability was evaluated for both extenders. A viability of 77.1% was for the semen in the control medium while a viability of 81.2% was for the semen in the experimental medium. At 48 hours from collection the semen in the control medium had a viability of 66.4% while the semen in the experimental medium had a viability of 69.5%.

At 72 hours from collection the semen in the control medium had a viability of 47.5% while the semen in the experimental medium had a viability of 56.4%.

At 96 hours from collection the semen in the control medium had a viability of 37.6% while the semen in the experimental medium had a viability of 42.3%.

At 120 hours from collection the semen in the control medium had a viability of 24.3% while the semen in the experimental medium had a viability of 29.1%.

The egg yolk is the most used non-penetrant cryoprotector for refrigerating the

buck semen, due to its protective effect on plasmatic and acrosomes membranes [16]. Due to interactions between this and the enzymes from the seminal plasma, such as the coagulating egg yolk enzyme (EYCE), was concluded that the concentration of egg yolk in the extender is a very important factor. There were noted a variety of results for concentrations between 2% and 20% egg yolk in the extender [1]. Our experiments proved that using a concentration of 5% egg yolk leads to better results comparatively to the concentration of 20% egg yolk for which the stage of centrifugation is necessary.

The glycerol is the most used cryoprotectant for preserving the semen at the farm animals because they obstruct the changes of phase of the extender when it is added in the dilution medium in concentrations under 3% [7]. Concentrations that are higher than 3% lead to a decrease survival during preservation and acrosomes damage with the result of fertility decrease [7]. Glycerol has an osmotic effect and a direct effect upon the plasmatic membrane by linking the phospholipid membranes.

Although the main effect of glycerol cryoprotection is manifested at extra-cellular level, it can enter into cell and remains bonded with the plasmatic membrane or in cytoplasm [2].

But the studies demonstrated that the glycerol, even though it has a cryoprotective effect, is metabolically toxic depending on concentration and the temperature at which it is added. The non-glycolitic metabolisation of glycerol leads to accumulation of certain toxic metabolites in cytoplasm [8]. The negative effect is linked to the osmotic impact produced by them, or to an irritating effect of it upon the mucous membrane of the inseminated females. After adding the glycerol, the cell rapidly contracts itself, the process being associated to the elimination of intracellular water, and after an ingression of glycerol follows a slow rebound at the initial volume. Adding glycerol in the semen dilution medium can cause changes to cellular membrane permeability, with negative effects on spermatic cell viability and motility and the increase of acrosomes damaging rate.

In order to eliminate the harmful effects caused by the use of glycerol, our experiments were focused on the use of an extender that does not contain glycerol. This was replaced with trehalose. Trehalose is a non-permeable cryoprotective, whose activity of protection is the result of the osmotic effect. The cryoprotective properties of the trehalose introduced in the extender used for semen preservation were confirmed also by other studies at various species (ram, bull, buck) [1].

The trehalose protection mechanism for cell against dehydrating or freezing presume the proteins and membranes stabilisation. Biological materials deterioration caused by refrigeration or freezing have two main causes:

- changes of the transition of phase of the membrane lipids
- changes of structure of the membranary proteins

The break of the hydrogen bond between water molecules and hydrofile regions of the phospholipids from the lipid double layer of the membrane, leads to an increase of aggregation degree of the lipid polar heads and, consecutively, of the acil chains from the structure of these phospholipids. The result is the transition of the lipids from the lamellar liquid phase to the gel phase [6]. If the transition takes place with defects of aggregation, at rehydrating can appear regions with defects of aggregation which leads to membranes rupture.

Crowe [6] suggested that in the process of replacing the water, the trehalose directly interacts with the hydrofile heads of the phospholipids, thus reducing the Van der Waals forces between the remains of acil hydrocarbonate and thus the integrity of the membrane remains intact after rehydration.

Our experiments demonstrated that trehalose added in the extender maintains the structural and functional stability of spermatic cell plasmatic membrane during the liquid preservation at 4 degrees C, similar results being obtained also in other experiments made on buck semen [1],[4].

CONCLUSIONS

1. The semen preserved in experimental extender on trehalose base and egg yolk (5%) registered a superior motility in contrast to the semen preserved in the control extender on egg yolk base (20%) and glycerol at 120 hours from collection.

2. The semen preserved in experimental extender on trehalose base and egg yolk (5%) registered a superior viability in contrast to the semen preserved in the control extender on egg yolk base (20%) and glycerol at 120 hours from collection.

3. The semen preserved in experimental extender on trehalose base and egg yolk (5%) registered a functional superiority of the membrane in contrast to the semen preserved in the control extender on egg yolk base (20%) and glycerol at 120 hours from collection.

4. The buck semen collected by electroejaculation can be preserved through refrigeration and used for artificial insemination for 120 hours when using an extender on trehalose base.

ACKNOWLEDGEMENTS

Research was carried out in the research project ADER 531 Maximizing progress Carpathian goat breed by implementing innovative biotechnology breeding in family farms, funded by MADR.

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