Comparative Examination of Deep-Frozen Ram Semen after Thawing and Incubating an Different Solution

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Abstract

Optimizing the cryopreservation technology of ram semen is a very important task. In our institution we try to improve the different stages of the cryopreservation procedure- the preparing (dilution and precooling), deep-freezing and thawing. In the literature review we compared the own preparing and deep-freezing methods with different methods, that were used by another authors. In our study we examined the quantity of ejaculates in spring and autumn, the changes of motility% and the capacitation status of ram semen samples after the thawing and incubation (39ºC, 2h) in different thawing and incubation solutions. The thawing and incubation solutions contain PBS (phosphate buffer solution) without- (control solutions) and with different addition materials (semen plasma, different ram's and ewe's blood serum). In our experiments we determined the changing of the motility%, the capacitation status and the rate of acrosome-reacted cells after the thawing and incubation (at 39ºC, 2h).

Keywords: cryopreserved ram semen, CTC fluorescent staining, capacitation, acrosome-reaction, decapacitation

1. Introduction

Our aim was optimize the deep-freezing and thawing methods of lacaune ram semen. In this study we examined the lacaune ram semen samples in different stages of cryopreservation. For example: preparing, deep-freezing, thawing and incubation. During the mentioned procedure the results of motility% and the capacitation status were worsened.Our aim is to improve these results (motility%, capacitation status) in deep-frozen and thawed and incubated (39ºC, 2h) ram semen samples. The thawing solutions which were used, contained ram semen plasma or different ewe's and ram's blood serum. We examined the different solution's effective the after thawing and incubating (at 39ºC, 2h). According to Salamon and Maxwell (1995/a) the glycerol is the most commonly used protective substance in diluents for freezing ram semen. For semen frozen by the slow „conventional" method, and using mainly hipertonic diluents, most investigators found that the optimalglycerol concentration was within the range of 6-8% spermatozoa frozen rapidly by the pellet method survived best with 3-4% glycerol in the diluents. Graham et al. (1978) reported that glycerol levels above 6% were detrimental to post-thawing survival of spermatozoa. It was suggested that the optimal glycerol concentration in diluted semen is also related to its final concentration relative to the spermatozoa. (Colas, 1975).The level of glycerol incubated in diluents for frozen storage of ram semen is ultimately limited by its toxicity (Fahy, 1986), which in turn depends on cooling and freezing rate, diluted composition and method of addition of glycerol.Examination of the combined effect of glycerol concentrations and cooling rates (0-8 % and pelleting on dry ice andinto liquid nitrogen,Visser and Salamon, 1974; 0-16% and cooling at 1-100ºC/min, Fisher and Fairful, 1984) showed that the higher the cooling rate, the lower the optimum glycerol concentration, and thebest post-thaw sperm survival rates observed by the later investigations were for 4-6% glycerol and freezing rate of 10-100ºC/min.

(Salamon and Maxwell, 1995/a) Salamon and Maxwell (1995/a,b) summarized the results of researchers, who had cryopreserved ram semen. They wrote about dilution, cryopreservation methods and insemination results (fertility%). The dilution that was used by Colas and Brice (1976) is similar to what we used, but they did not cryopreserve the ram semen in straw. We cryopreserved it in pellets and diluted in two steps. In the first step we used a diluent solution, that content lactose and egg yolk. (Diluent 1). In the second step we added glycerol to diluent as a cryoprotective matterial. (Diluent 2) We added the diluent 2 to the diluted ejaculates at 4-5ºC. The glycerol concentration was 4% in the dilution 2.Colas and Brice (1976) added the dilution with cryoprotective matterial at 4ºC. In their experiments the dilution ratio 1:5 was used. We also had similar dilution ratio 1:5 used. However the dilution ratio in different ejaculates sample was different. This is dependent on the density of ejaculates. Maxwell et al. (1980) used dilution with different compositions. They had frozen the ram semen in pellets. The quality of thawed semen was controlled with insemination experiments (fertility %) by Colas and Brice (1976) and Maxwell et al. (1980). These dilution and deep-freezing methods are similar to the methods we used. Gomez et al.(1997) incubated fresh and cryopreserved ram semen samples at different temperatures (30 °C, 39 °C) for 0.5-19hours.They determined the rate of acrosome-reacted cells in ram semen samples that were incubated at 39°C and 30°C incubation temperatures.

2. Materials And Methods

The place of experiments was Pharmagene-Farm Ltd. in Mosonmagyarovar.
The date of experiments was spring and autumn of 2009.
The samples were taken from lacaune 6 rams.

Measured parameters:
- Quantity and quality of ejaculates (ml)
- Determination of motility% with light microscope
- We determined the proportion of cells with intact membrane, the capacitated cells and acrosome-reacted cells in different phase the preparation for deep freezing and after the thawing and incubation (39ºC, 2 h) with the CTC fluorescent staining method.

2.1 Preparation

2.1.1 Method of dilution

-I. diluent contains lactose and egg yolk. I. dilution was added to the fresh semen on ram's body temperature (39ºC).
-II. diluent contains lactose, egg yolk and glycerol. II. Diluent was added to the diluted semen on 4ºC. (Dilution with glycerol on 4ºC)

2.2 Phases of preparation for deep freezing and thawing

- Dilution with I. diluent on body temperature (39ºC)
-Cooling to 20 °C, 1 hour
- Cooling to 4 °C 1.5 hour
- Dilution with II. Diluent on 4 °C
-Equilibration (1-2 hour)
- Freezing in pellets on dry ice
- Freezing in liquid nitrogen

2.3 Composition of thawing solution

- PBS (phosphate buffer solution)
- PBS + semen plasma
- PBS + blood serum of different rams and ewes

Tests were carried out in several phases.

2.4 The phases were following:

1. 20°C
2. 4°C (Before the equilibration)
3. 4°C (After the equilibration)
4. Thawed and incubated (39ºC, 2 h) samples in different solutions

- Control thawing solution: PBS (Phosphate buffer solution)
- Solution 1 (Thawing solution with decapacitation factors): PBS (Phosphate buffer solution) + ram semen plasma
- Solution 2 (Thawing solution with decapacitation factors): PBS (Phosphate buffer solution) + ewe's blood serum (ear number: 8181)
- Solution 3 (Thawing solution with decapacitation factors): PBS (Phosphate buffer solution) + ewe's blood serum (ear number: 8211)
- Solution 4 (Thawing solution with decapacitation factors): PBS (Phosphate buffer solution) + ram's blood serum (ear number: 4245)
- Solution 5 (Thawing solution with decapacitation factors): PBS (Phosphate buffer solution) + ram's blood serum (ear number: 4056)
- Solution 6 (Thawing solution with decapacitation factors): PBS (Thawing solution with decapacitation factors) + ram's blood serum (ear number: 4012)

2.5 CTC fluorescent staining

We applied the CTC fluorescent staining method, which was published by Gillian et al. (1997) to determine the rate of membrane intact cells, capacitated-, and acrosome-reacted cells. The CTC-fluorescence assay used in this study to assess the capacitating state of the ram spermatozoa. The CTC staining solution was freshly prepared prior to each experiment and contained CTC-HCl (750µM, Sigma) in a stock buffer (stored 4°C) comprising Tris (Trizma base 20mM, Sigma), NaCl (130mM) and L-cysteine (5mM) that had been filtered using a 2.0 µm filter and adjusted to pH 7.8. The solution was protected from light and warmed prior to use to prevent cold-shock. At room temperature a 45µL sample of spermatozoa suspension ($100x10^6$ sperm mL^{-1}) was placed in light-protected Eppendorf tube and equal volume of CTC staining solution was added. After thorough mixing for 30 s, a 10µL sample of filtered glutaraldehyde (EM Grade; 1% v/v in Tris, pH 7.8) was added to fix the cells in suspension. A 10 µL sample of this uniformly mixed suspension was placed on to a clean microscope slide and 10 µL of 1, 4-diazabicyclo [2.2.2]-octane (DABCO, 0,22M, Sigma) dissolved in glycerol: phosphate buffer saline (9:1; stored below 0°C) was added to retard photo-bleaching. A cover slip was placed on the sample, excess fluid was removed by compression and the edges of the cover slip were sealed with colourless nail varnish.

All samples were processed in triplicate and experiment was replicated four times. A total of 200 spermatozoa per slide were assessed within 3 h of preparation under a x100 objective on an Olympus BHS microscope fitted with phase contrast and fluorescent optics. The excitation beam was passed through a 405 nm-band pass filtered and CTC emission was observed through a 455 nm diachronic mirror with an additional 375 nm barrier filter. The spermatozoa were allocated to one of three staining categories: F, with uniformed head fluorescence, which is thought to be representative of incapacitated, acrosome-intact cells: B, bright acrosome fluorescence only, representative of capacitated, acrosome-intact cells, and AR fair head fluorescence or an irregular banded pattern, representative of acrosome-reacted cells. Bright fluorescence was observed on the mid-piece of the spermatozoa in all staining categories. Spermatozoa which were viewed side-on or displayed patterns other than those described were excluded from this classification. (*Gillian et al.*, 1997).

2.6 Method of assessment of results

2.6.1 Changing of the capacitation status (decapacitation)

Capacitating is a reversible process; however the acrosome-reaction is irreversible. The rate of capacitated cells must be reduced and the rate of cells with intact membrane must be increase as a result of the decapacitation process. After the thawing the decapacitation process has occurred, if the rate cells with intact membrane was increased and the rate of capacitated cells was reduced in the thawing solution with different decapacitation factors. The capacitation, decapacitation and the acrosome-reaction are continuous and dynamic processes.

2.6.2 Determination of the rate of decapacitation

The rate of cells with intact membrane must increase at least as much as the rate of capacitated cells decrease otherwise the rate of acrosome-reacted cells is increasing.

Table 1: **Change of the cell's rate in different category**

Change of the cell's rate in different category			
Process	**Cells with intact membrane**	**Capacitated cells**	**Acrosome-reacted cells**
Capacitation	reduce	increase	unchanged
Decapacitaion	increase	reduce	unchanged
Acrosome-reacted cells	unchanged or reduce	reduce	increase

3. Results And Discussion

3.1 Quantity of ram semen 2009

Already in the 2007-2008 was carried outon the basis oftest results, it was found that therams of the season, inautumnal samples takenin the springmorethan thatamount. The quality of the autumnal samples under three years of data is also greater than the previous one.

Table 2: Quantity of ram semen 2009 (ml)

Quantity of ram semen 2009 (ml)						
Date of collecting	**ENAR number of rams**					
	4245 n=10	**4056 n=10**	**4012 n=10**	**4045 n=10**	**23386 n=10**	**23144 n=10**
Average 2009. spring	1,2	1,5	1,1	1,3	1,4	0,8
Average 2009. autumn	1,2	2,0	1,7	1,5	1,5	1,3
Average spring and autumn	**1,2**	**1,6**	**1,3**	**1,4**	**1,5**	**1,0**

3.2 Effect of semen plasma and blood serum to thawing and incubating solution

Added semen plasma and blood serum to thawing and incubating solution have also observed that the effect on the thawed samples after cryopreservation upon the motility%. The thawing solutions contained ram semen plasma and different ewe's and ram's blood serum. We examined the effect of this material for motility% after the thawing.

3.3 Effect of decapacitation factors using for rate of motility%

According to our observation the decapacitation factors improve of motility%in different samples. The use of semen plasma in thawing solution usually influences the motility% positively. However the blood serum as a decapacitation component has not always proved effective. The use of blood serum as decapacitative component is highly specific.

Table 3: **Effect of semen plasma and blood serum on motility% in post-thawing ram semen samples**

Effect of semen plasma and blood serum on motility% in post-thawing ram semen samples after the thawing (2009)						
Thawing solutions	Semen samples (ram's ear number)					
	4245 n=6	4056 n=3	4012 n=3	4045 n=3	23386 n=3	23144 n=3
Motility% in 39ºC	68%	69%	68%	65%	58%	60%
Control thawing solution: PBS (phosphate buffer solution)	23%	19%	18%	23%	15%	25%
Thawing Solution 1.: PBS (phosphate buffer solution) + semen plasma	23%	22% (+3%)	13%	38% (+15%)	23% (+8%)	30% (+5%)
Thawing solution 2.: PBS (phosphate buffer solution) + ewe's blood serum (ear number 8181)	16%	9%	0%	Na.	25% (+10%)	Na.
Thawing solution 3: PBS (phosphate buffer solution) + ewe's blood serum (ear number 8211)	18%	14,38%	0%	Na.	15%	Na.
Thawing solution 4: PBS (phosphate buffer solution) + ram's blood serum (ear number 4245)	18%	15%	0%	Na.	20% (+5%)	Na.
Thawing solution 5.: PBS (phosphate buffer solution) + ram's blood serum (ear number 4056)	20%	20% (+1%)	16%	30% (+7%)	20% (+5%)	20%
Thawing solution 6.: PBS (phosphate buffer solution) + ram's blood serum (ear number 4012)	20%	10%	1,50%	32,5% (+9,5%)	32,5% (+17,5%)	Na.
Evaluation increased motility%						Control samples
						10%-20%
						5%-9,9%
						1%-4,9%

3.4 Incubation

After thawing the motility% of cryopreserved semen samples was 30-35%. The results on motility% in incubated (39ºC, 2 h) semen samples were in contrast 20-27.5%.

Table 4: **Results of incubation test on 39°C, 2 hours (Motility %)**

Results of incubation test on 39 ºC, 2 hours (Motility%)						
	Semen samples (ram's ear number)					
	4245 n=5		4012 n=5		23386 n=5	
Before deep freezing	70%		70%		70%	
Incubation test (39ºC, 2h)	Before	After	Before	After	Before	After
Control thawing solution: PBS (phosphate buffer solution)	25%	20%	30%	20%	20%	12,5%
Thawing Solution 1.: PBS (phosphate buffer solution) + semen plasma	30%	25%	35%	27,5%	25%	5%
Thawing solution 2.: PBS (phosphate buffer solution) + ewe's blood serum (ear number 8181)	10%	5%	25%	7,5%	7,5%	2%
Thawing solution 3: PBS (phosphate buffer solution) + ewe's blood serum (ear number 8211)	20%	12,5%	17,5%	10%	17,5%	15%
Thawing solution 4: PBS (phosphate buffer solution) + ram's blood serum (ear number 4245)	25%	22,5%	30%	27,5%	30%	22,5%
Thawing solution 5.: PBS (phosphate buffer solution) + ram's blood serum (ear number 4056)	32,5%	17,5%	15%	15%	20%	10%
Thawing solution 6.: PBS (phosphate buffer solution) + ram's blood serum (ear number 4012)	30%	10%	25%	15%	32,5%	22,5%
Evaluation	Control samples' motility%					
	Higher motility%, than control samples'.					

3.5 Result of different samples capacitation status

Table 5: **Effect of semen plasma and blood serum on decapacitation in ram semen samples after thawing**

Effect of semen plasma and blood serum on decapacitation in ram semen samples after the thawing						
Thawing solutions	Semen samples (ram's ear number)					
	4245 n=6	4056 n=3	4012 n=3	4045 n=3	23386 n=3	23144 n=3
Control thawing solution: PBS (phosphate buffer solution)	0%	0%	0%	0%	0%	0%
Thawing Solution 1.: PBS (phosphate buffer solution) + semen plasma	1,5% **23%** **21%** 2% 15%	**20%** **29%** 6%	**26%** **22%**	8% 5,5%	**24%** **30%**	0%
Thawing solution 2.: PBS (phosphate buffer solution) + ewe's blood serum (ear number 8181)	**20,5%** 9,5%	**40%**	9,5% 6,5%	Na.	**23%**	0%
Thawing solution 3: PBS (phosphate buffer solution) + ewe's blood serum (ear number 8211)	**40%**	7,5% **39,5%**	7,5% 10,5%	5,50%	4%	0%
Thawing solution 4: PBS (phosphate buffer solution) + ram's blood serum (ear number 4245)	19% 0,5% 19,5% 16,5% 4%	2,5% **29%**	**20%** 2,5%	Na.	Na.	Na.
Thawing solution 5.: PBS (phosphate buffer solution) + ram's blood serum (ear number 4056)	2% **21%** 2,5% 4% 16,5% 1%	6% 12%	13% **22%**	Na.	11,5% 6,5%	0%
Thawing solution 6: PBS (phosphate buffer solution) + ram's blood serum (ear number 4012)	12,5% **24%** 7% 7%	14% **31,5%**	5,5% 17%	Na.	Na.	0%
Evaluation rate of decapacitated cells (%)						Control samples
					20%<	

According the results established, the thawing solution with semen plasma almost without exception is effective in all cases, but the decapacitation efficiency thawing solution with blood serum shows a rather high standard deviation. Using the appropriate blood serum can substantially increase the efficiency of thawing solution. Using blood serum and decapacitation factors in the practice of livestock farming can be applied after cross matching. This may be increased to variety of animals' frozen-thawed semen's quality. It would be worthwhile to undertake further analysis in a laboratory of the composition used ewe's and ram's blood serum, particularly its HCO_3^- and Ca^{2+}concentrations. These ions play an important role in capacitating and acrosome-reaction. This is also very important the fatty acids and amino acids composition.

3.6 Conclusion

The quantity and quality of ram semen samples were better in autunm than in spring. The ram semen samples are more suitable for cryopreservation in autumn.The use of semen plasma and blood serum in thawing solutions was absolutely effective, however the use of blood serum is highly specific. The ram semen plasma proved to be more efficient than blood serum. Thawing solution „completed" ram semen plasmas almost always improves the motility% and capacitating status after the thawing and incubation tests (39°C, 2h).The important components in blood serum are Ca^{2+}, HCO_3^- ions, hormones, fatty acids and amino acids. The most important components are Ca^{2+}, HCO_3^- ions, because of their important role in capacitating and acrosome-reaction.

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