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**THE DEVELOPMENT OF RAM SEMEN DEEP-FREEZING  
METHODS BASED ON THE RESULTS OF PHASE  
EXPERIMENTS**

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## 1. INTRODUCTION

Until today, the deep-freezing of ram semen has not been unequivocally accomplished. Insemination with the help of thawed seminal deep-frozen using different techniques in artificial straw and pellet is in many cases only possible through surgical or semi-surgical ways, using the laparoscope method to get the semen into the horns of the uterus. Insemination carried out in such a manner can yield results if the seminal has 35-40% of motility%. However, for a successful cervical-uterine insemination carried out using the insemination catheter, seminal having 60-65% of motility% is required. Producing this is a very complicated task. It is enough if only a minor error occurs during deep-freezing, and the entire day's work is wasted. When producing deep-frozen semen, one must pay attention to the exact composition of thawing solution, its proper quantity, making sure that pre-cooling is carried out in several steps; moreover, one must also exercise caution when adding the thawing solution containing the cryoprotective material, as well as make sure to observe the equilibration time, the deep-freezing speed and the thawing of the material at appropriate temperatures and in solutions of appropriate composition. The reproductive capacity of the spermatozoa, however, does not depend on the number of motility% alone. In many cases, the living, moving cells have already undergone the so-called capacity-like deformations, or acrosome reaction, the premature occurrence of which render them unsuitable for

insemination. Let us not forget, however, that it is crucial to insemination that these activities take place.

During our investigations, the author examined these deformations with the help of the CTC (chlorine-tetracycline-hydrochloride) fluorescent dying procedure. This made it possible in the case of the samples examined to clearly establish the ratio of cells with an intact membrane that have undergone capacity-like deformations and the acrosome reaction. This thereby makes it possible for us to pinpoint the deep-freezing phase in which the spermatozoa get damaged, and also which steps need to be changed. During thawing, it becomes evident which composition of thawing solution is most suitable for reversing – that is, decapacitating – the capacity-like deformation that occurred during deep-freezing. Apart from the inspections above, we also carried out incubation tests (at 39°C, 2 h) in order to examine the vitality of ram semen thawed after deep-freezing.

Apart from examination the deep-freezing methods, it is also crucial to raise, keep, feed, and regularly train proper rams in a stock that are capable of producing semen suitable for deep-freezing; furthermore, the quality of the acquired seminal must be continuously monitored and the results recorded so that during the investigation it turns out which specimen's seminal is suitable for deep-freezing. We must also pay attention to the time of year, for example the autumn high season or the spring expletive season. In author's experience, the animals produce seminal most suitable for deep-freezing in late autumn. In

case of the thawing solutions with different compositions, we examined their decapacitating effect.

## **2. MATERIAL AND METHOD**

During the examinations carried out at Pharmagene-Farm Ltd. between 2007 and 2009, the author examined the quantity and quality of spring and autumn ram semen, as well as its suitability for cooling down to 2-4°C and for deep-freezing with the involving of seven lacaune rams and a single buck. The amount of fresh semen acquired from the rams, the number of living cells in it, the number of living cells in the deep-frozen and thawed semen, as well as the ratio of spermatozoa that underwent capacitation-like membrane modification and acrosome reaction were determined in certain phases of the deep-freezing procedure. During the investigations, the efficiency of different decapacitating solutions was examined by living cell percentage determination, by examining the change in the ratio of cells that underwent capacitation-like deformation and those with intact membranes, as well as by employing incubation experiments, all of which served to examine the vitality of spermatozoa after thawing.

### **2.1. Animals involved in the inspection**

#### **2.1.1. Rams**

- 299

- 4012
- 4045
- 4056
- 4245
- 23144
- 23386

### **2.1.2. Buck**

- Jimmy 001

### **2.1.3. Tests and inspections**

1. Inspecting the percentage change in living cells, the membrane and acrosome deformations, and the reproducing capacity during the cooling of ram semen cooled at 2-4 °C, based on data from autumn 2007.
2. Ram semen phase inspection, membrane and acrosome deformation inspection during preparation, deep-freezing and thawing, based on data from spring 2008.
3. Inspecting the effect of the change of seasons on the suitability for deep-freezing, based on data from spring 2008 and autumn 2008.



4. Inspecting the effect of thawing solutions containing decapacitation factors on the percentage of living cells and on decapacitation, as well as application during heat exhaustion tests, based on data from 2009.

### **3. RESULTS AND EVALUATION**

#### **3.1. Inspecting the percentage change in living cells, the membrane and acrosome deformations, and the reproducing capacity during the cooling of tup semen cooled at 2-4 °C, based on data from autumn 2007**

In autumn 2007, we examined seminal that was freshly acquired, diluted, and stored at 2-4°C. The investigations included the determination of the number of living cells using a light microscope, the determination of the ratio of cells that underwent capacitation-like deformation or acrosome reaction with an intact membrane using the CTC fluorescent dying method, as well as testing the seminal of different tups while using them for insemination. During this latter examination, we separately evaluated the success of insemination using the seminal acquired, which surprisingly yielded better results than the seminal used during the everyday routine. Insemination was carried out using the cervical-uterine method and a Milovanov type insemination catheter. The seminal used was freshly taken, diluted tup semen, as well as tup semen cooled at 2-4°C. Insemination was carried out twice a day: in the mornings and in the afternoons.

### 3.1.1. Motility% results of diluted seminal cooled at 2-4°C, year 2007

During the investigation, the living cell percentage was first determined immediately after the semen was acquired, then after 1 and 2 days of storage at 2-4°C.

The results are included in the table below.

*Table 1:: Changes of motility % in ram semen on 2 - 4 °C storage*

Changes of motility % in ram semen on 2 - 4 °C storage						
Ram's ear number	4012	4045	4056	4245	Average	Standard deviation
Storage	Motility %					
Fresh semen	76,15%	77,00%	76,15%	77,27%	76,64%	0,58%
1st day	70,00%	67,50%	61,67%	71,00%	67,54%	4,18%
2nd day	55,00%	60,00%	55,00%	60,00%	57,50%	2,89%

As a result of the investigation, it can be ascertained that out of the four samples examined and following 2 days of storage at 2-4°C, the based on the mean average of the living cell percentage in the seminal of two rams, namely that of #4045 and #4245, a result of 60% was measured, which means that generally speaking, the seminal is suitable for artificial insemination after two days of storage at 2-4°C.

### **3.1.2. Examination tests results of cell membrane and acrosome states in cooled (at 2-4°C) and diluted semen, year 2007 inspection results**

During the cell membrane and acrosome inspection carried out using the CTC fluorescent dying method, however, the seminal taken from animal number 4056 proved to be the best. However, comparing the two investigations, we can ascertain that cervical-uterine insemination using the seminal from ram #4056 is only recommended following 1 day of cooling at 2-4°C. In case of the average values for rams #4012 and #4045, insemination is also recommended after maximum 1 day of cooling at 2-4°C. However, based on the average values of sample #4245, insemination using the freshly diluted semen is recommended because according to the fluorescent investigations of the membrane, the seminal taken from ram #4245 resisted cold storage at 2-4°C the least. In this case, if the semen is stored and cooled, then decapacitating factors must be added to the solution during the dilution process. The most straightforward thing is direct sperm plasma addition in a ratio of 20 V% before dilution, which can also be used in case of the other samples in order to improve the results. Furthermore, the addition of a higher ratio of carbohydrate to the attenuant can be recommended, in the form of fructose, lactose or gum arabic.

### **3.1.3. Successful insemination percentage using diluted semen cooled at 2-4°C, year 2007 results**

The percentage of successful inseminations carried out within the stock using diluted ram semen cooled at 2-4°C, not for experimental purposes and not on oestrus synchronized ewes was 61.59% in the year 2007. It is worth noting that in case of experimental insemination and using oestrus synchronized ewes, this ratio is 70.58%. This is probably a result of lower population and higher than usual attention and oestrus synchronization. From the aspect of successful insemination, the seminal from rams #4245, #4056 and #299 proved to be the most effective. However, in case of experimental insemination, the seminal from rams #4012 achieved a significantly better result. All in all, we can say that the above-average results can be considered as good. And in case of experimental insemination, the additional caution and attention yielded even better results.

### **3.2. Ram semen phase inspection, membrane and acrosome deformation examination during preparation, deep-freezing and thawing, based on spring 2008 data**

When evaluating the results, we considered both the motility% – by which we mean living and moving cells – and the condition of the membrane and the acrosome, as each of these characteristics greatly contribute to insemination capacity.

The results demonstrate that the semen taken from the he-goat named Jimmy 001 endured deep-freezing the best, with an average ratio of 64.8-61.15% of cells with intact membranes. Let us not forget, however, that the starting value before deep-freezing was also the highest with 81%.

From the aspect of intact membrane, the semen taken from animals #4245, #23386, and #4012 also endured deep-freezing well. #4056 and #23144 also reacted well to decapacitating factors.

### **3.2.1. CTC fluorescent staining procedure**

The diagrams called “Percentage of spermatozoa with intact membrane in each phase of deep-freezing” and “Percentage of spermatozoa that underwent capacitation in each phase of deep-freezing into pellet”, demonstrate the effect that the decapacitating factors (sperm-plasma, blood-plasma) added to the PBS solution had on the semen taken from the rams and the single buck, following the thawing of the samples after deep-freezing.

The suitability of the semen taken from the rams and the busk for deep-freezing is ranked according to the results. The best result during thawing was achieved by the semen from the ram #299 ENAR. Of all the samples, this one gave the best reactions to both decapacitating factors.

After having been melted back in PBS, it took the last spot among the samples with intact membrane with a result of 29.5%. However, with

the help of decapacitating factors, the ratio of samples with an intact membrane is 77% and 66%, respectively.

Rams #299, #4012, #4056, #4045 and #23386 reacted in the best way to sperm-plasma, as a component containing a decapacitating factor, although it should be noted that in case of the diluted seminal from ram #23386, the ratio of spermatozoa with an intact membrane was not far behind with the blood-plasma added to the PBS, either: it was only 1.5% lower than the results from melting back using PBS + sperm-plasma.

Using blood serum as a component containing a decapacitating factor to melt back the samples in PBS, the diluted semen from the aforementioned specimen #23386 and #23144 yielded the better results.

The seminal taken from the buck called Jimmy 001 and thawed after deep-freezing demonstrated a bad reaction to both decapacitating factors because in this particular case, the presence of components containing decapacitating factors in the solution used for thawed slightly reduced the ratio of cells with intact membranes. It is understandable in this particular case, as semen plasma and blood serum from a different species, the sheep, was added to the PBS solution. Surely we would have achieved better results if we had used buck blood plasma. Regardless, it can be ascertained that even despite this fact the ratio of cells with intact membranes is relatively high: it is the second best when using PBS for thawing; the third in the solution

with added blood serum; and takes the 5<sup>th</sup> spot when semen plasma was added to it, out of a possible 8. It should be mentioned, however, that in the 20°C phase, the ratio of intact membranes was the highest in Jimmy 001's sample, namely 81%.

Regarding the ratio of spermatozoa, it should be mentioned that components containing decapacitating factors – especially in case of semen plasma -, it is sufficient to take a look at the diagrams, and we can see that the ratio of spermatozoa that have been capacitated decreases just as the ratio of those with an intact membrane increases. Therefore, it seems that solutions containing the decapacitating factor take effect. At the following rams, this is clearly visible in the diagrams: for #4012, #4045, #4056, #23386, #299 ENAR, the decapacitating factors of the semen plasma take a clearly visible effect; for tup #23144, the diagram shows the positive effect of the decapacitating factors found in the blood serum.

In all cases, the number of acrosome reactions significantly increased after the thawing following deep-freezing.

### **3.2.2. Light microscope inspections**

In case of the light microscope inspections, we can estimate the reproduction capacity based on the number of living and moving cells in the diluted and thawed ram semen after deep-freezing.

When determining the reproduction capacity of the individual ejaculates, first of all we evaluated the ratio of living and moving cells

based on the light microscope inspections, and then defined the ratio of spermatozoa with an intact membrane that had undergone capacitation and acrosome reactions using the fluorescent staining method. The joint application of these two inspection methods may render help in checking the suitability of deep-frozen ram and buck semen for insemination.

However, proper feedback can only be gained from insemination results and from the so-called non-return index and the parturition percentage in particular.



*Table 2. : Motility % of diluted ram and buck semen samples before cryopreservation and after thawing*

ram/buck	Date of cryopreservation 2008	Diluted sample on 20°C	Phosphate buffer solution (PBS)	Phosphate buffer solution (PBS) + semen plasma	Phosphate buffer solution (PBS) + blood serum
ENAR number (ear number)	Motility %				
4012	2008.02.26	70-75	15	25	20
	2008.03.04	70	30	30	30
	2008.03.12	70	25	35-40	40
	2008.03.25	70	25	30	20
4045	2008.02.26	75-80	20	25	25
	2008.03.06	70	30	35-40	25
	2008.03.12	65	60	40	25
4245	2008.02.27	70	20	30	25
4056	2008.02.27	70	25	30	30
	2008.03.04	70	35-40	35	30
	2008.03.20	70-75	35	40-45	40-45
23144	2008.03.19	70	15	25	30-35
23386	2008.03.19	65	15	20	25
299	2008.03.20	65	15	25	20
Jimmy 001	2008.03.11	65	20	45	25

With regard to the motility%, ram #4056 achieved outstanding results after thawing, which reached 35-40% of motility%, or even 40-45% in some cases, with the addition of decapacitation factors, which is a

good result in case of deep-frozen ram semen. This seminal can surely be used.

The living cell ratio in the deep-frozen buck semen was around 35-50%. With #4045, the number of living cells after thawing was 45-40% and even 60% in an individual case.

Before analysing the data from the light microscope inspections, it should be noted that the movement of ram semen that has undergone capacitation quickens, meaning that by observing a larger number of living and moving cells under the microscope, it is possible that the ratio of spermatozoa that have undergone capacitation also increases. This could affect the results to a small degree.

### **3.3. Inspecting the effects of season change with regard to the suitability for deep-freezing, based on data from spring and autumn 2008**

Inspecting the effects of season change took place in spring and autumn 2008. During the investigation, the amount of individual ejaculated was determined along with the motility%; after deep-freezing, we also examined the membrane and acrosome states after thawing in PBS and in solutions containing various decapacitating factors.

### **3.3.1. Results of inspecting the effects of season change**

- The results of phase inspections carried out on ram semen taken in autumn and spring, then deep-frozen and thawed reveal that autumn semen are of better quality and more suitable for deep-freezing.
- There is seasonality in case of rams as well, which depends on the climate, the hemisphere and the latitude, as according to the investigations carried out by Ismaya (2003), as days become longer in the southern hemisphere, the quantity of semen produced by rams increases and its quality improves. In contrast, the breeding high season in the northern hemisphere is in autumn, when the days are getting ever shorter. The results of the investigations carried out at Pharmagene-Farm Ltd. also support the idea that autumn ram semen is of better quality. This investigation justifies that there is indeed seasonality among rams.

### **3.3.2. Recommendations regarding the inspection of the effects of season change**

- In our opinion, further tests should be run in various months of the year in order to further support the notion that there is seasonality among rams as well. We recommend inspecting motility%, semen quantity, semen density, cell membrane and acrosome condition in freshly acquired semen.

- A further recommendation is to record climate conditions in various months of the year, especially when semen is collected. The observation and recording of climate conditions should be evaluated together with the quality of ram semen.
- Density measurement data should also be added in order to get a clearer picture about the effect of season change and climate conditions on semen production.
- In the future, the Kovács-Foote dying method should also be employed apart from the CTC fluorescent staining procedure because this method provides further information about the ratio of living/dead cells, about motility and about the ratio of cells that have undergone an acrosome reaction, as well as ones that have a missing or damaged acrosome.

### **3.4. Year 2009 results**

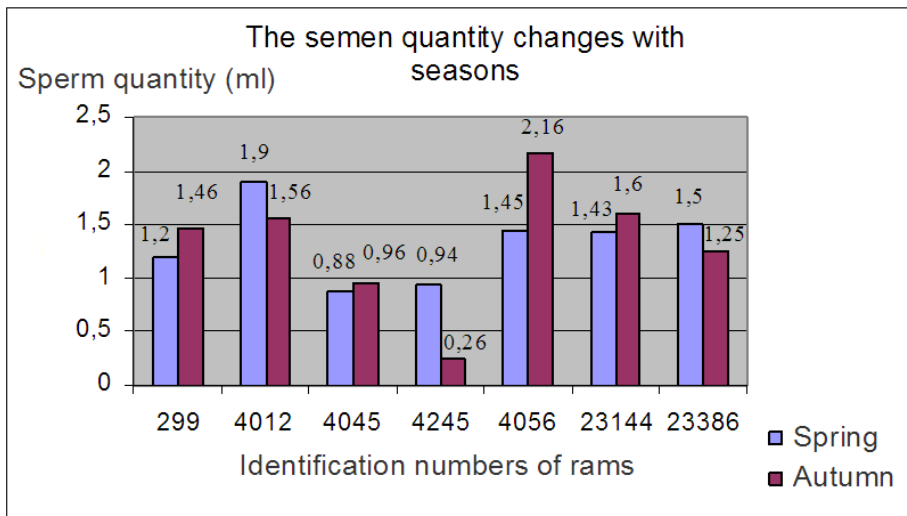
#### **3.4.1. Ram semen quantity, year 2009 results**

We could determine based on the inspection carried out in 2008 already that the quantity of semen taken from rams is greater in the high season, i.e. autumn, than in spring. According to data from two years, the quality of autumn semen is also better.

Table 3: Chnages of average semen quantity in different seasons (ml)

Chnages of average semen quantity in different seasons (ml)							
Seasons/ENAR number	299	4012	4045	4245	4056	23144	23386
Spring	1,2	1,9	0,88	0,94	1,45	1,43	1,5
Autumn	1,46	1,56	0,96	0,26	2,16	1,6	1,25

Diagram 1: Chnages of average semen quantity in different seasons (ml)



**3.4.2. The effect of thawing solutions containing decapacitating factors on the motility% and on decapacitation, as well as the application of incubation tests (at 39°C, 2 h), based on year 2009 data**

According to our observations, decapacitating factors also affect the motility% in samples that are thawed after deep-freezing. Generally speaking, solutions containing decapacitating factors have a positive effect on motility%; but in our peculiar case it is not all the same which animal's blood serum is used in the thawing solution in conjunction with another animal's seminal as a material containing decapacitating factors.

### 3.4.3. The inspection of decapacitation after thawing in different decapacitating solutions

Table 4: Effect of decapacitation factors for rate of live cells in semen samples after the thawing (2009)

Effect of decapacitation factors for rate of live cells in semen samples after the thawing (2009)						
Thawing solutions	Semen samples (ram's ear number)					
	4245	4056	4012	4045	23386	23144
Live cells rate in 39°C	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 increasing rate of live cells (%)						Control samples
						10%-20%
						5%-9,9%
						1%-4,9%

*Table 5: Effect of thawing solution with different decapacitation factors for rate of decapacitated cells after the thawing*

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



Based on the results above, it can be ascertained that the thawing fluid containing sperm plasma is effective in almost every case. However, the efficiency of blood serum taken from different animals shows a rather big spread. Yet, by using the adequate blood-plasma, the efficiency of decapacitating solutions can be greatly improved. In practice, they can be applied in a certain farm based on preliminary “cross-tests”. It is advisable to make blood serum using blood serum taken from all the rams in use and from several ewes, to be used with the decapacitating solutions, and carry out preliminary tests. This could help to increase the decapacitating effect of solutions prepared for the thawed seminal taken from different species.

In the future, a laboratory analysis of blood serum composition, with special regard to  $\text{HCO}_3^-$  and  $\text{Ca}^{2+}$  ion concentration should be taken into consideration, as these ions play an important role in capacitation and in acrosome-reactions. Furthermore, the fatty acid and amino acid composition of the solutions could also turn out to be really important.

#### **3.4.4. Incubation experiment results, year 2009**

After thawing, samples with a motility% of 30-35% showed results of 20-27.5% following 2 hours of incubation at 39°C. These samples are suitable for laparoscope insemination. The results from the year 2009 incubation experiments show that for the time being, deep-frozen and thawed samples are only conditionally suitable for cervical-uterine insemination, for experimentation purposes alone. It should be noted, however, that the results of ram semen deep-frozen and thawed in

2008 are much better compared to the year 2009 results. This could be due to weather change, but further analysis and test need to be carried out in order to draw a conclusion.

*Table 6: Results of motility % during the incubation test on 39 °C, 2 hours*

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

Based on the results above, the following conclusions can be made:

The use of blood semen and semen plasma containing decapacitating factors as an additive in thawing solutions is absolutely justified. However, the use of decapacitating factors with added blood serum is also highly specific. On the other hand, semen plasma improves the number of motility% in samples and the extent of decapacitation in virtually every case during incubation following thawing after deep-freezing.

When inspecting the decapacitating process, the inspection of materials containing decapacitating factors – such as examining the composition of blood serum from different animals: ewes and rams – is also crucial in order to develop the optimal composition of re-heating solutions containing decapacitating factors. In this case, ions playing a relevant role in capacitation ( $\text{Ca}^{2+}$ ,  $\text{HCO}_3^-$ ) could be important parameters, along with the concentration of various hormones and fatty acid and amino acid composition.

It is recommended to continue with the investigations, as well as use the Kovács-Foote staining method to carry out further staining and evaluation, then compare this data with the results yielded by the CTC fluorescent method and the motility%. The Kovács-Foote method provides results about both the movement ability and the acrosome condition. The CTC fluorescent staining procedure provides information only about the membrane and the acrosome. The motility% is the result of a separate light microscope examination;

therefore, it cannot be ascertained whether a cell that has undergone an acrosome reaction is capable of moving or not. On the other hand, the advantage of the CTC fluorescent staining procedure is that it can also be used to examine the decapacitation process. With the joint application of these two staining methods, not only can we acquire more information concerning the quality of the sample examined, but it could also give further ideas for the development of examination methods used to determine the insemination suitability of the semen.

#### **4. NEW SCIENTIFIC RESULTS**

1. In the lacaune ram semen samples deep-frozen in pellet, the occurrence of the decapacitation of the cells in the control thawing solution (PBS, phosphate buffer solution) can be determined by the examination in ratio of cells that have undergone capacitation compared to the ratio of cells that have undergone capacitation and have an intact membrane, as well as by the inspection of the increase in the number of cells with intact membranes. Providing that in the a thawing solutions containing decapacitating factors (PBS + sperm plasma, PBS + blood plasma), the ratio of cells with intact membranes simultaneously increases compared to the cells present in the control PBS solution with intact membranes or that have undergone capacitation, together with a drop in the ratio of cells that have undergone decapacitation, then we can talk about decapacitation.

2. The extent of decapacitation can be determined by comparing the increase in the ratio of cells with intact membranes found in solutions containing added decapacitating factors (PBS + sperm plasma + blood plasma) to the ratio of cells that have undergone capacitation and have an intact membrane present in the thawing solution containing a phosphate buffer (PBS), functioning as a control solution; on the condition that the ratio of cells that have undergone capacitation-like deformation is reduced by at least the same extent as the increase in the ratio of cells with intact membranes.
3. In case of lacaune ram semen samples deep-frozen in pellet and then thawed, when examining the effect of the thawing solution with added semen plasma containing a phosphate buffer on the number of living cells and the capacitation condition, we found that the re-heating solution with added sperm plasma containing a phosphate buffer had a positive effect on lacaune ram semen samples deep-frozen in pellet and then thawed regarding both the ratio of living and moving spermatozoa and their capacitation condition.
4. As a result of the investigations, it can be ascertained that blood seum used as an additive in the thawing solution could increase the motility% and improve the decapacitation

condition in case of the lacaune ram semen thawed after deep-freezing in pellet. However, the effect of the blood serum from various animals is highly specific. Completely different effects per specimen can be attained.

5. The effect of the blood serum added to the solution used for thawing after deep-freezing on the motility% in the lacaune ram semen, as well as on the capacitation state depends on the composition of blood serum from different animals. It is highly probable that it is mostly influenced by  $\text{Ca}^{2+}$  and  $\text{HCO}_3^-$  concentration, as these ions play a role in the occurrence of capacitation and the acrosome reaction.
6. It can be ascertained that semen plasma created from ejaculates collected either in spring or in autumn can be used in thawing solutions as natural additives containing decapacitating factors. In contrast, Domingez et al. (2008) state that only semen plasma created from semen taken in autumn is an effective decapacitating additive.
7. Based on our inspections, it can be ascertained that in the ejaculate of lacaune rams, the percentage of cells that have undergone an acrosome reaction is higher in autumn than in spring.

## **5. SCIENTIFIC PUBLICATIONS AND LECTURES DEALING WITH THE SUBJECT MATTER OF THE DISSERTATION**

### **Reviewed publications issued in scientific literature in English language**

**Csiba Anita** – Gyökér Erzsébet– Gergátz Elemér- Gombkötő Nóra: Effect of semen plasma and blood serum for motility % and capacitation status of cryopreserved and thawed ram semen – The Experiment Journal 2015., Vol. 34(4), 2150-2161 (ISSN 2319-2119)

**Csiba Anita** – Gyökér Erzsébet– Gergátz Elemér- Gombkötő Nóra: Comparative examination of deep-frozen ram semen after thawing and incubating in different solution - International Journal of Applied Science and Technology Vol. 5. No. 6. 2015.12.31. (ISSN 2221-0997)

### **Reviewed publications issued in scientific literature in Hungarian language**

Szabados Tamás – Gergátz Elemér – Gyökér Erzsébet – **Csiba Anita** – Gyimóthy Gergely – Németh Attila – Mihályfi Sándor (2008): A külső méhszáj alakulások és a termékenyítő katéter bejuttathatóságának vizsgálata lacaine juhállományban. - Állattenyésztés és Takarmányozás. 57. 1. 55-64. p.

## **Reviewed publications issued in scientific literature in Hungarian language in academic journal**

Szabados Tamás – Gergátz Elemér – Gyökér Erzsébet – Németh Attila – Mihályfi Sándor – **Csiba Anita** - Gyimóthy Gergely (2008): Az ejakulátumok mennyiségének vizsgálata lacaune kosoknál. - Acta Agronomica Óváriensis., 50. 2. 67-78. p.

## **Conferences**

### **International conference, lecture in English language**

Gergátz Elemér – Gyökér Erzsébet – Csiba Anita – Németh Attila: Quality of the deepfrozen buck semen produced in pellet form and mini-straw – European Regional Conference on Goats 2014 7-13 April 2014 Debrecen – Hungary, Oradea – Romania 88 p.

### **Hungarian Conferences, lectures in Hungarian language**

**Csiba Anita** – Németh Attila – Mihályfi Sándor (2008): Kos- és bakspermiumok kapacitációszerű elváltozásainak és akroszóma-reakcióinak vizsgálata fluoreszcens festési eljárás segítségével. - XXXII. Óvári Tudományos Nap – Élelmiszergazdaságunk Kérdő Jelei Napjainkban Nemzetközi Konferencia, Mosonmagyaróvár, 2008. október 9. (ISBN 978-963-9883-05-5 Konferencia kiadvány)



**Csiba Anita** - Gyökér Erzsébet - Németh Attila - Mihályfi Sándor (2009): Őszi és tavaszi kossperma mélyhűthetőségének összehasonlítása – XV. Ifjúsági Tudományos Fórum, Keszthely 2009. április 16. (ISBN 978-963-9639-33-1)

**Csiba Anita** - Gergátz Elemér - Gyökér Erzsébet - Németh Attila - Mihályfi Sándor: Tavaszi és őszi kossperma mennyiségének, minőségének, valamint mélyhűthetőségének összehasonlítása - LIII. Georgikon Napok nemzetközi tudományos konferencián elhangzott előadás – 2011. szeptember 29. (ISBN 978-963-9639-44-7 kiadvány 187-197. p.)

**Csiba Anita** – Gergátz Elemér: Kossperma mélyhűtés módszereinek összefoglaló vizsgálata - LIV. Georgikon Napok nemzetközi tudományos konferencián elhangzott előadás – 2012. október 7-8. (ISBN 978-963-9639-48-5 kiadvány 112-125 p.)

## **Poster publications**

### **Poster publication in English language**

**Csiba Anita** - Gergátz Elemér: Improvement the cryopreservation of ram semen based on the result of phase experiments – Pannon Egyetem Georgikon kara által rendezett LV. Georgikon Napok, 2013. szeptember 26-27. (ISBN 978-963-9639-52-2 kiadvány 39p.)

## **Poster publications**

### **Poster publicationa in Hungarian language**

**Csiba Anita** – Németh Attila – Mihályfi Sándor (2008): A fluoreszcens spermafestési eljárás bemutatása – poszter XXXII. Óvári Tudományos Nap – Élelmiszergazdaságunk Kérdő Jelei Napjainkban Nemzetközi Konferencia, Mosonmagyaróvár, 2008. október 9. (ISBN 978-963-9883-05-5 Konferencia kiadvány)

**Csiba Anita** - Gergátz Elemér - Gyökér Erzsébet: Kossperma mélyhűtésre való alkalmasságának vizsgálata minőségének megőrzése és ellenőrzése a mélyhűtés különböző fázisaiban poszter- XXXIV. Óvári Tudományos Nap Magyar Mezőgazdaság – lehetőségek, források, új gondolatok c. konferencia, 2012. október 5. (ISBN-978-963-9883-93-2 kiadvány 355-362 p.)