ISSN-2319-2119

RESEARCH ARTICLE
Csiba, A et al, The Experiment, 2015., Vol. 34(4), 2150-2161

EFFECT OF SEMEN PLASMA AND BLOOD SERUM FOR MOTILITY % AND CAPACITATION STATUS OF CRYOPRESERVED AND THAWED RAM SEMEN

Abstract:

The quality control involved utilising various methods of frozenram semen and optimising the cryopreservation technology, which are both very important tasks. The experiments were carried out using light microscopy and CTC fluorescent staining on the various stages of ram semen samples preparing for cryopreservation procedure, thawing and incubating (at 39 ºC, 2 hours) in the various compositions of solution with and without decapacitation factors. These three types of tests gave the results, which allows for the determination of the suitability of the semen for insemination. Experience has shown that variety rams for used for propagating deep-freezing semen samples are very different. The results demonstrated the necessity of using decapacitation factors. In our study the results of controlling at different stages of freezing and thawing (i.e. decapacitation process) and incubation experiments were analysed, and compared with the results, that have already been published in this field.

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

1. Introduction:

Our aim was optimize the freezing methods of lacaune ram semen. In the experiments we examined the lacaune ram semen in different stages for example: preparing, deep freezing, thawing, and incubating. During the mentioned procedures the capacitation process occurred. Capacitation is a reversible process with decapacitation factors. The thawing solutions which were used, contained ram semen plasma, ewe's and ram's blood serum. These solutions potentially incurde capatitation factors. We examined the solution's effective for motility% and capacitation status after the thawing and incubating (on 39ºC, 2 hours).

2. Materials and Methods:

The place of experiments was Pharmagene-Farm Ltd. in Mosonmagyarovar.

The date of experiments was spring and autumn of 2009.

In the experiments we examined the lacaune ram semen. The semen samples, semen plasma protein and blood serum were taken in spring and autumn. The semen samples and plasma proteins were taken from 6 rams.The blood serum taken from 3 rams and 2 ewes.

2.1 Measured parameters

We measured quantity and quality of ejaculates (ml) and determination of motility% with light microscope. (The results of examinations are in Table 4.) We determined the proportion of uncapacitated cells with intact membrane, the capacitated cells with intact acrosome 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. (The results of

THE EXPERIMENT
INTERNATIONAL JOURNAL OF SCIENCE AND TECHNOLOGY
ISSN-2319-2119
RESEARCH ARTICLE
Csiba, A et al, The Experiment, 2015., Vol. 34(4), 2150-2161

examinations of decapatitation are in Table 5. The results of examination of incubation are in Table 6.)

2.2 Method of dilution

I. diluent contains lactose and egg yolk. The 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 4◦C)

2.3 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

2.4 Composition of thawing solution

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

Tests were carried out in several phases.

2.5 The phases were following:

1. 20°C
2. 4°C (Before the equilibration)
3. 4°C (After the equilibration)
4. Thawed samples in different thawing 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)

THE EXPERIMENT
INTERNATIONAL JOURNAL OF SCIENCE AND TECHNOLOGY
ISSN-2319-2119
RESEARCH ARTICLE
Csiba, A et al, The Experiment, 2015., Vol. 34(4), 2150-2161

- 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)

2.6 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 capacitation state of the ram spermatozoa was modified from that previously described by *Sailing and Stroey* (1979) and *Ward and Storey* (1984) for mouse 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 with in 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)

THE EXPERIMENT
INTERNATIONAL JOURNAL OF SCIENCE AND TECHNOLOGY

ISSN-2319-2119

RESEARCH ARTICLE
Csiba, A et al, The Experiment, 2015., Vol. 34(4), 2150-2161

2.7 Method of assessment of results

2.7.1 Decapacitation

Capacitation 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.

2.7.2 Determination of the rate of decapatitation

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'srate 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
De-capatitaion	increase	reduce	unchanged
Acrosome-reacted cells	unchanged or reduce	reduce	increase

3. Results and Discussion:

3.1 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 not always provided effective. The use of blood serum as decapacitative component is highly specific.

The important components in blood serum are Ca^{2+}, HCO_3^- ions, hormons, fatty acids and amino acids. The most important components are Ca^{2+}, HCO_3^- ions because of their important role in capacitation and acrosome-reaction. However we will examined these ions and another components in our next studies.

3.2 The results of motility %

Nts'emelo et al. (2010)'s results of motility% is better than our own results before the deep freezing and after the thawing. We used for deep freezing and thawing semen samples for lacaune rams. Nts'emelo et al. (2010) used semen from another sheep breeds like merino, pedi and zulu.

THE EXPERIMENT
INTERNATIONAL JOURNAL OF SCIENCE AND TECHNOLOGY

ISSN-2319-2119

RESEARCH ARTICLE
Csiba, A et al, The Experiment, 2015., Vol. 34(4), 2150-2161

Table 2: Effect of different thawing solutions for motility % for different semen samples from lacaune, merino, pedi, zulu rams before the preparation for freezing and after the thawing

Effect of different thawing solutions for motility % for different semen samples from 6 lacaune rams after the thawing (2009)	
Phases of before the preparation for deep freezing and after the thawing	Motility% of semen samples from 6 lacaune rams (ear numbers of rams: 4245, 4056, 4012, 4045, 23386, 23144)
Motility % in 39ºC before the preparation for deep freezing	64.66 ± 6.66%
After the thawing in different solution	
Control thawing solution: PBS (phosphate buffer solution)	20.50 ± 5.50%
Thawing Solution 1.: PBS (phosphate buffer solution) + semen plasma	24.83 ± 11.83%
Thawing solution 2.: PBS (phosphate buffer solution) + ewe's blood serum	16.66 ± 7.66%
Thawing solution 3: PBS (phosphate buffer solution) + ewe's blood serum	15.79 ± 1.41%
Thawing solution 4: PBS (phosphate buffer solution) + ram's blood serum	17.66 ± 2.66%
Thawing solution 5.: PBS (phosphate buffer solution) + ram's blood serum	21.00 ± 5.00%
Thawing solution 6.: PBS (phosphate buffer solution) + ram's blood serum	19.30 ± 17.80%

Effect of different thawing solutions for motility % for different semen samples from merino, pedi zulu rams before the preparation for deep freezing and after the thawing (Nts'emelo et al., 2010)		
Sheep breeds	Motility % of different sheep breeds of semen samples in 39ºC before the preparation for deep freezing	Motility % of different sheep breeds of semen samples after the thawing
Merino	75.80 ± 1.50 %	38.3 ± 4.2 %
Pedi	76.70 % ± 1.90%	26.7 ± 2.8 %
Zulu	75.00 ± 2.30 %	45.8 ± 1.9 %

THE EXPERIMENT
INTERNATIONAL JOURNAL OF SCIENCE AND TECHNOLOGY

ISSN-2319-2119

RESEARCH ARTICLE
Csiba, A et al, The Experiment, 2015., Vol. 34(4), 2150-2161

3.3 Effect of different thawing solution for membrane and acrosome status of ram semen samples after the thawing

Gillian et al. (1997) observed the effect of cryopreservation of the capacitation status of ram spermatozoa.

Table 3: Comparing of the result of our own experiments with results in literature

Comparing of the average eresults of own experiments with average results inliterature				
Membran and capacitation status of cell membrane	Fresh semen		Frozen-thawedsemen	
	Our own result	Results in literature	Our own result	Results in literature
"F" pattern (uncapacitated cells with intact membrane)	80%	61.3% (Gillian et al, 1997)	48.29%	6.7 % (Gillian et al., 1997)
"B" pattern (capacitated cells)	10.37%	no data	33.92%	65.9% (Gillian et al, 1997)
"AR" pattern (acrosome-reacted cells)	9.36%	16% (Gomez et al., 1997)	17.6%	25.9% (Gillian et al. , 1997)

Based on the data of Table 3 considering:

- The rate of uncapacitated cells with intact membrane in the fresh and frozen-thawed semen samples were better in own examination than in published results by Gillian et al. (1997).
- The rate of capacitated cells in the frozen-thawed ram semen samples much more lower in results of your experiments than published results by Gillian et al. (1997).
- The rate of acrosome-reacted cells (“AR” pattern cells) before the freezing and after the thawing were better than published that results by Gillian et al. (1997) and Gomez et al. (1997)

Their results demonstrate, that cryopreservation may cause membrane changes in ram spermatozoa functionally equivalent to capacitation. The frozen-thawed ram semen samples were thawed in thawing solution with semen plasma and blood serum as a result of addition these solutions the motility % and capacitation status will be better after the thawing and incubation.

THE EXPERIMENT
INTERNATIONAL JOURNAL OF SCIENCE AND TECHNOLOGY

ISSN-2319-2119

RESEARCH ARTICLE
Csiba, A et al, The Experiment, 2015., Vol. 34(4), 2150-2161

Table 4: Effect of different thawing solution for rate of decapacicated cells in lacaune ram's deep frozen semen samples after the thawing

Effect of different thawing solution for rate of decapacicated cells in lacaune ram's deep frozen semen samples after the thawing					
Thawing solutions	Semen samples (lacaune ram's ear number)				
	4245	4056	4012	4045	23386
Thawing Solution 1.: PBS (phosphate buffer solution) + semen plasma	12.5±10.5%	18.3±12.3%	24.0±2.0%	6.25±1.25%	27.0±3.0%
Thawing solution 2.: PBS (phosphate buffer solution) + ewe's blood serum (ear number 8181)	15.0±5.5%	40.0±0.0%	8.0±1.5%		23.0±0.0 %
Thawing solution 3: PBS (phosphate buffer solution) + ewe's blood serum (ear number 8211)	40.0±0.0%	23.5±16.0%	9.0±1.5%	5.5±0.0%	4.0±0.0%
Thawing solution 4: PBS (phosphate buffer solution) + ram's blood serum (ear number 4245)	11.9±11.0%	15.75±13.25%	11.25±8.75%		
Thawing solution 5.: PBS (phosphate buffer solution) + ram's blood serum (ear number 4056)	7.83±6.83%	9.00±3.00%	17.5±4.5%		9.0%±2.5%
Thawing solution 6.: PBS (phosphate buffer solution) + ram's blood serum (ear number 4012)	12.625±5.625%	22.75±8.75%	11.25±5.75%		

THE EXPERIMENT
INTERNATIONAL JOURNAL OF SCIENCE AND TECHNOLOGY

ISSN-2319-2119

RESEARCH ARTICLE
Csiba, A et al, The Experiment, 2015., Vol. 34(4), 2150-2161

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.

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 capacitation and acrosome-reaction. This is also very important the fatty acids and amino acids composition.

3.4 Incubation

Maxwell et al. (1999) examined the fresh and frozen-thawed spermatozoa incubated in phosphate buffer solution (PBS) and phosphate buffer solution (PBS) with 20% seminal plasma.

They found the motility % and capacitation status were greater in phosphate buffer solution (PBS) added with 20% seminal plasma after the incubation (6h, 37ºC). There were more "F" pattern and "AR" pattern cells in this solution. According to Maxwell et al. (1999) demonstrating that this seminal plasma had inhibited capacitation-like changes and improved motility of spermatozoa

Table 5: Average motility % after thawing before and after incubation (at 37 and 39ºC, 2h)

Average motility % after the thawing before and after incubation			
	Incubation temperature and time	Motility % before incubation after the thawing	Motility % after incubation
Results of own experiments	Incubationat 39ºC, 2h	25%	17.5%
Results of Mataveia et al.'s experiments (2010)	Incubationat 37ºC, 2h in phosphate buffer solution (PBS) + 20 % semen plazma	39%	26%

THE EXPERIMENT
INTERNATIONAL JOURNAL OF SCIENCE AND TECHNOLOGY

ISSN-2319-2119

RESEARCH ARTICLE
Csiba, A et al, The Experiment, 2015., Vol. 34(4), 2150-2161

Average motility % after thawing before and after incubation in different solution			
Incubation temperature and time	Incubation solution	Motility % before incubation	Motility % after incubation
Incubationat 39ºC, 2h (results of own experiments)	Controlsolution PBS	25 ± 5 %	16.25 ± 3.75 %
	PBS + ewe's bloodserum (earnumber: 8181)	16.25 ± 8.75 %	4.25 ± 2.75 %
	PBS + ewe's bloodserum (earnumber: 8211)	18.75 ± 1.25 %	12.5 ± 2.5 %
	PBS + ram's bloodserum (earnumber: 4245)	27.5 ± 2.5 %	25 ± 5%
	PBS + ram's bloodserum (earnumber: 4056)	23.75 ± 8.75 %	13.75 ± 3.75 %
	PBS + ram's bloodserum (earnumber: 4012)	28.75 ± 3.75 %	16.25 ± 6.25 %
	PBS + semen plasma	30 ± 5 %	16.25 ± 11.25 %
Incubation at 37ºC, 2h (Mataveia et al., 2010)		35 ± 3.04 %	28.9± 3.04%

The incubation temperature that we used was higher than Mataveia et al. used in 2010. Therefore the motility % was lower than their results. We used 39ºC incubation temperature because it is the body temperature of ewe's. We want to modeled the ewe's body temperature. However the thawing at 37ºC showed better results than thawing at 39ºC.

3.5 Statistical analysis

3.5.1 Motility %

The two factors variance analysis showed highly significant differences (SD=4.4%) between effect of difference composition thawing solutions for motility% of the cryopreserved ram semen samples from different rams after the thawing.

Based on the above fact that the different thawing solutions for example buffer solution without or with 20% semen

THE EXPERIMENT
INTERNATIONAL JOURNAL OF SCIENCE AND TECHNOLOGY
ISSN-2319-2119
RESEARCH ARTICLE
Csiba, A et al, The Experiment, 2015., Vol. 34(4), 2150-2161

plasma or blood serum from ewes or rams have a different effect for the motility% different ram semen samples.

As a result of the statistical analysis justied, that the effect of thawing solution with 20% semen plasma has significant difference from effect of the kontrol thawing solution (phosphate buffer solution without added matterial) and the thawing solution (phosphate buffer solution) with ram's and ewe' blood serums. (SD=0.06516)

There are significant difference between effects of control thawing solution (phosphate buffer solution) and the effect of phosphate buffer solution with another 20% added matterials, for example ram semen plasma, ewe's blood serum (SD= 0.108358586; SD=0.8927677) and the ram's blood serum SD= 0.075555556).

In 95% probably level is a low significant differences can be detected between the different thawing solution with different added materials for example ram semen plasma, ram's and ewe's blood serum. ($F_{95\%}$= 3,23; F=3.663150697)

After the statistical analysis the results show that the 20% ram semen plasma as added matterial to phosphate buffer thawing solution has absolutely positive effect for motility%, but this positive effect showed significant differences between different ram's semen samples.

3.5.2 Rate of semen cells with intact membrane in different samples in different thawing solution

In 95% probably level is a low significant differences can be detected between effect for rate of cells with intact membrane of different thawing solutions with different composition. ($F_{95\%}$=2.19)

In 95%, and 97,5% probably levels are significant differences between the effects of different composition thawing solutions. ($F_{95\%}$=3.18; F97.5%=3.97; F=4.870189799)

We can concluded that the effects of thawing solution that contain has a significant different with effect for the motility% of control solution (SD= 7,166567) and phosphate buffer thawing solutions with ewe's (SD=11.41666667; SD= 8.952380952) and ram's blood serum. (SD= 10.04901961)

The rate of semen cells with intact membrane in the different semen samples that thawed in the belowed thawing solutions showed significant different with samples that in thawing solution without (control) and with added ram semen plasma.

There are no significant differences between the rate of cells with intact membrane after the cryopreservation in different containing thawing solutions.

3.6 Conclusion

The use of semen plasma and blood serum in thawing solutions was effective for the increase the motility% after the thawing, however the use of blood serum is highly specific. The ram semen plasma proved to be more efficient than blood serum after thawing. Two ram's blood serums were effective in raising of the motility% after the thawing. Incubating solution added with ram's blood serum improves the motility during the incubation test (39ºC, 2h)

In the thawing and incubating solutions with ram's blood serums were more efficient to increase the motility% and rate of uncapatitated cells with intact membrane of ram semen cells than thawing and incubating solution with ewe's blood serum.

Motility% results of Mataveia et al. (2010) and Nts'emelo et al. (2010) were better in thawing solution with semen samples after the thawing than our results. Nts'emelo examined another sheep breeds like merino, pedi, zulu.

Gillian et al. (1997) and Mataveia et al (2010)'s motility results of deep freezing ram semen after the thawing were better than motility % results of our own experiments.

However the results of our own experiments the membrane and capacitation status were better than other authors for example Gillian et al. (1997) and Gomez et al. (1997).

In our experiments result's the rate of uncapacitated cells with intact membrane is so much higher in fresh and frozen-thawed ram semen samples than the results that published by Gillian et al. (1997) and Gomez et al. (1997).

The rate of capacitated and acrosome-reacted cells in our experiments was lower than the results of Gillian et al. (1997) and Gomez et al. (1997).

4. References:

1. *Gillian, L. – Evans, G. – Maxwell, W.M.C.* (1997): Capatitation status and fertility of fresh and frozen-thawed ram spematozoa – Reprod. Fertil. Dev., 2997. 9. 481-7
2. *Nts'emelo-Mahoete (2010):*In vitro embryo and semen cryopreservation in sheep – doctoral dissertation – Magister Science Agriculturae- in the Faculty of Natural Agricultural Sciences, 2010, May 54-57p.
3. *Mataveia, G. A. - Terblanche, S. J. – Nöthlin, O. J.- Gerber, D.* (2010): Effect of heterologous seminal plasma and semen extenders on motility of frozen–thawed ram spermatozoa - Tydskr. S. Afr.vet.Ver. (2010) 81(3): 139–142
3. *Maxwell, W.M.C. – Evans, G. - Mortimer, S.T. - Gillian, L. - Gellatly, E.S. – McPhie, C.A.*(1999): Normal fertility in ewes after cervical insemination with frozen-thawed spermatozoa supplemented with seminal plasma – Reprod. Fertil. Dev. , 1999. 11. 123-126
4. *Mendoza, N. - Casao, A. - Pe´rez-Pe´,R. - Jose´, A. - Cebria´n-Pe´rez, J. A. andMuino-Blanco* T. (2013): New Insights into the Mechanisms of Ram Sperm Protection by Seminal Plasma Proteins - Biology of Reproduction (2013) 88(6):149, 1–15 Published online before print 1 May 2013.DOI 10.1095/biolreprod.112.105650
5. *Pérez-Pé, R. - Cebrián-Pérez, J. A. and Muiño-Blanco* T. (2001): Semen plasma proteins prevent cold-shock membrane damage to ram spermatozoa - Departamento de Bioquímica y Biología Molecular y CelularFacultad de Veterinaria, Zaragoza, Spain - Received 18 July 2000; accepted 7 March 2001. Available online 12 October 2001. – www.sciencedirect.com
6. *Sailing, P. M. – Storey, B. T.* (1979): Mouse gamete interactions during fertilization in vitro. Chlortetracycline as a fluorescent probe for the mouse sperm acrosome reaction Cell Biology 83. 544-55
7. *Ward, C. R. – Storey, B. T.* (1984): Determination of the time course capacitation in mouse spermatozoa using a chlortetracycline fluorescence assay. Dev. Biol. 104. 287-96

THE EXPERIMENT
INTERNATIONAL JOURNAL OF SCIENCE AND TECHNOLOGY

ISSN-2319-2119

RESEARCH ARTICLE
Csiba, A et al, The Experiment, 2015., Vol. 34(4), 2150-2161

Authors & Affiliation

Csiba, A. [1*] – Gyoker, E. [1] – Gergatz, E.[1]-Gombkoto N.[2]

[1] Pharmagene Farm Ltd., Biotechnical Station University of West Hungary

[2] University of West Hungary Faculty of Agricultural and Food Sciences

Mosonmagyarovar, Janossomorjai ut 4, 9200 (ZIP code), Hungary