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THE LITHUANIAN TECHNIQUE OF BULL SEMEN COLLECTION AND CRYOPRESERVATION

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Abstract

Semen is diluted if spermatozoa motility is not lower than 70% and spermatozoa concentration is no less than 800 million per millilitre. For semen evaluation, plastic bags with the ejakulate are stored in the termostat at 27±1 °C. Good quality semen is diluted in a plastic bag at a ratio of 1:1. 15 to 20 minutes after collection of the last ejaculate, semen is diluted for the second time. The finitial concentration should be no less than 15 million of motile spermatozoa per dose after the thawing. After the second dilution the semen is packaged in sterile plastic straws. Thereafter then semen should be cooled at 4±2 °C for 180 to 240 minutes. Semen is frozen in a biocontainer with a special equipment – a cooper freezing net. The temperature should be maintained at –150±30°C depending on the individual characteristics of bull semen. The freezing cycle should last for 8 minutes incuding temperature stabilization.

Keywords: bull, semen, collection, dilution, cooling, freezing.

Introduction

The Lithuanian technique for bull semen cryopreservation is one of the most outstanding achievements of the Lithuanian Institute of Animal Science. Creative workers in numerous institutes, plants and enterprises of the former USSR took part in the development of the technique under the leadership of prof. P. Pakėnas. New advances in cryobiology, chemistry and the other fields of science were used in the development and improvement of individual technological elements.

Two major stages in the development of the technique can be singled out. In 1958–1972, separate operations of semen collection and preparation were integrated into a single technological process. Optimum semen packaging form, i.e. a polymeric tube, has been found, devices for semen preparation and cryopreservation as well as insemination tools were designed.

In 1973–1990, simplification and mechanization of various semen preparation processes were carried out. Semen packaging and straw printing machines were produced, special equipment for semen coding and freezing was designed and optimum conditions for semen dilution, cooling and freezing were determined.

The Lithuanian technique is currently employed by more than 40 enterprises in Lithuania and in the states of the former USSR. The Lithuanian technique has some advantages over the other techniques. The optimum size of the straw ensures steady semen freezing process, and maximizes the usage of nitrogen tanks. The straws are handy. The equipment and materials used for the Lithuanian technique are much cheaper than those of French or German-origin.

This study presents technological processes and some research data of the Lithuanian technique.

Semen collection from breeding bulls

The quality and volume of ejaculates are mainly dependent on the ancestral features of bulls, their feeding, management frequency of usage preparation for semen collection and collection itself. The quality and quantity of the collected semen have a significant influence on the stocking up of the planned quantity of semen doses, progress in the purposeful

breeding activities for the improvement of the performance of the succeeding generation of animals, conception rate of cows and heifers and financial standing of the enterprise.

On the eve of semen collection, the bulls are washed under shower with water not below 18 °C and mild toilet soap or green soap is used. On the day of semen collection, the bulls are exercised about one hour. The exercise is carried out in double circular corridors, one of which is narrow (80 cm in width, 170 cm in height) for younger bulls and the other is wider (100 cm in width, 180 cm in height) for mature bulls, or in other buildings. Immediately before semen collection, the bulls are extra cleaned at a special site. After the treatment, the bulls are dried with sterile napkins of pressed cotton and led in the groups one after another in a circle to get them excited and mounting on the sire in front. Bullbreeders walking inside the circle should watch that the bull's penis would not touch the skin of the mounted animal. Only when heavy erection of the penis is evident, and the secretion from the accessory genital glands washes the urinogenital canal, the bulls are led to the collection area. After the first ejaculation, the bull is again led in a circle for about 12 to 15 minutes with the aim of stimulating his sexual activity and conditioning for the second ejaculation. If the third ejaculation is performed, then the interval is increased from 15 to 20 minutes.

Semen evaluation and dilution

Semen is diluted if spermatozoa motility is not lower than 70% and spermatozoa concentration is 800 million per millilitre (Table 1).

Table 1. Minimum requirements for fresh semen

Item	Requirement
Colour and appearance	White liquid viscous mass, sometimes with yellowish or greenish tint
Volume, no less than (ml)	1.5
Sperm motility, no less than (scale numbers; %)	7; 70
Sperm density, no less than (milliard/ml)	0.8
pH	6.8...7.4
Level of pathogenic spermatozoa*, no more than (%)	25
Including	
with pathologic heads, no more than (%)	20
other pathologic forms, no more than (%)	15
Coli titre	1:10
Nonpathogenic microbial count, no more than (thou./ml)	5
Pathogenic microorganisms (bacteria, viruses, fungi)	Should not be detected
Toxicogenic fungi	Should not be detected

* The level of pathogenic spermatozoa is investigated on worsening of other parameters of fresh semen

For semen evaluation, plastic bags are stored in the thermostat at 27±1 °C.

Semen of a good quality is diluted in a plactic bag with a continuous action syringe at a ratio of 1:1. The optimal temperature of the diluent (Table 2) being 27±1 °C (Fig. 1).

After dilution with the diluent of 27±1 °C, spermatozoa motility after freezing and thawing was by 6.0% ($P<0.05$), number of live spermatozoa by 8.0% ($P<0.01$) and number of intact acrosomes by 3.2% ($P<0.005$) higher if compared with the physiological parameters of semen diluted at 32±1 °C. After 5 hours exposure to 38±0.5 °C spermatoza motility after freesing and thawing was by 44.4% ($P<0.001$) higher. Absolute survival indicator and survival time were by 31.9% ($P<0.001$) and 7.6% ($P<0.05$) higher, respectively. The percentage of live spermatozoa was higher when the semen was diluted with the diluent of 25±1 °C and 20±1 °C.

However, the increased percentage was statistically insignificant in comparison with the semen diluted using dilution of 32±1 °C and 27±1 °C.

Table 2. Diluents for semen dilution and freezing

Components	LGY[1] (Nagase-Niwa)	LGSY[2]
	Rate	
Lactose (g)	11.5	10.5
Sodium citrate (g)	–	0.2
Glycerol (ml)	5.0	5.0
Egg yolk (ml)	20.0	15.0
Spermosan-3 (thou. u.)	50.0	50.0
Spermosan-PPK (thou. u.)	50.0–100.0	50.0–100.0

[1] LGY – lactose-glycerol-yolk
[2] LGCY – lactose-glycerol-sodium citrate-yolk

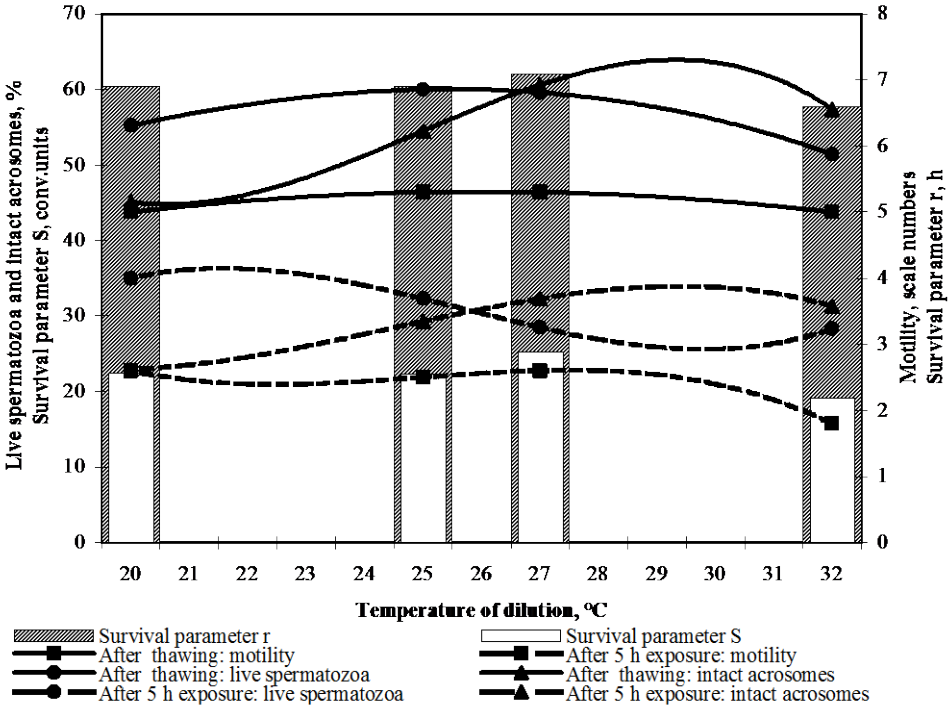

Figure 1. Physiological parameters of semen diluted at different temperatures

Afterwards, the bags are put into a separate beakers and kept on the table at 18–20 °C. In the course of 15 minutes, the temperature of the diluted semen lowers gradually to 18–20 °C (Fig. 2). The recorded ejaculate is diluted at a ratio 1:1 and laid up.

15 to 20 minutes after collection of the last ejaculate, semen is diluted for the second time, the temperature of the diluent being 18 to 20 °C. The diluent is poured in a small portion along the wall of the plastic bag and mixed up.

Semen is diluted to the spermatozoa concentration of no less than 15 million of motile spermatozoa in one straw after thawing. Sometimes the semen from high-class pedigree bulls may be diluted to the spermatozoa concentration of no less than 10 million of motile spermatozoa in a dose after thawing. But in this case, care should be taken not to worsen the conception rate of cows. After the second dilution semen is packaged.

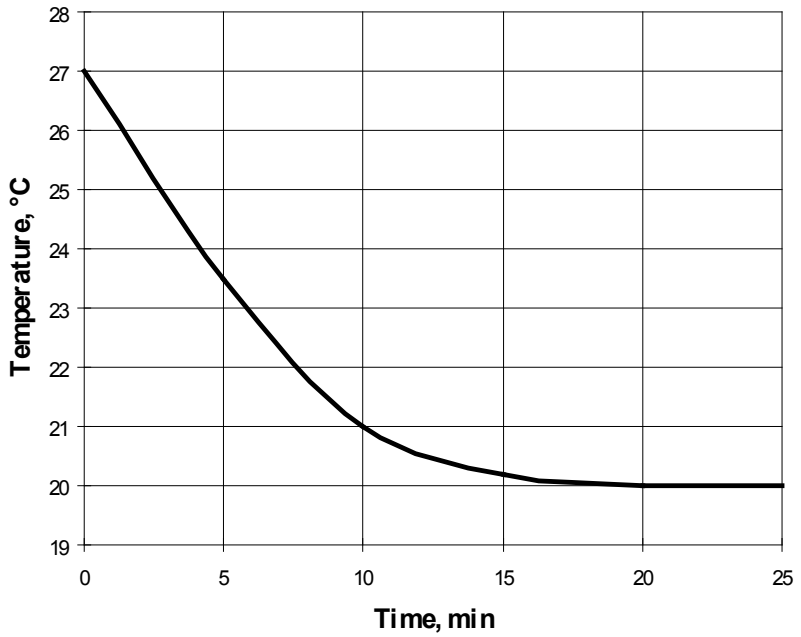

Figure 2. Sperm temperature vs. time after primary dilution (ambient temperature 20 °C)

Semen packaging in the straws

After the second (final) dilution, the semen is packaged in sterile and harmless polypropylenic straws (straw length 100 mm, outside diameter 2.4 mm, portion volume 0.27 ml) and sealed with coloured glass balls using the filling and sealing M6-APA model machine. During semen packaging, care should be taken to keep the air bubble in the straw between 5 to 12 mm and the glass balls pressed in 1.5 to 2 mm from the straw end. During freezing the semen column expands and if the air bubble was smaller, the pressure could push the ball out of the straw.

After packaging the air bubble should be transferred to the middle of the straw. The straw is taken down and shaken once or twice like a clinical thermometer, to transfer the air bubble to the middle of the straw.

Printing of straws. The printing machine with the ink-jet printing unit is used. Straws are marked with the name of the breeding enterprise, bull's name, registration number and breed. If necessary, other data can also be identified from a marked straw.

Semen cooling

Thereafter the semen should be cooled. The straws filled with the semen and sealed with the glass balls are spread on stainless steel racks containing up to 143 straws. For the apportionment of the straws, the racks are placed over the racking platform and the straws are rapidly spread with the flat of the hand. The balls pressed into straws enlarge their ends, therefore, by spreading the straws strictly in one row small gaps between them appear. This is normal.

The racks are stacked up. Up to 5 straw racks are accommodated in a box and held in a refrigerator at 4±2 °C. The cooling rate of semen depends on the number of doses in a box (Fig. 3). The physiological parameters of semen became worse if the cooling rate of semen was lower in comparison with the higher cooling rate, which, however, did not exceed the recommended limit of 0.25–0.5 °C/min. If the semen was cooled without using a box, the average cooling rate was 1.6 °C/min., and in this case the physiological parameters of semen were higher than those for semen cooled at a rate of 0.29 °C/min. In both cases, the diluent used was composed by lactose, glycerol, sodium citrate and yolk. The motility of spermatozoa cooled at a rate of 1.6 °C/min. to the temperature of 4±2 °C was by 10.4% ($P<0.01$) and after

5 hour exposure to 38±0.5 °C the absolute survival indicator was by 37.4% (P<0.001) and survival time by 20.7% (P<0.001) higher in comparison with semen cooled at a rate of 0.29 °C/min. (Fig. 4).

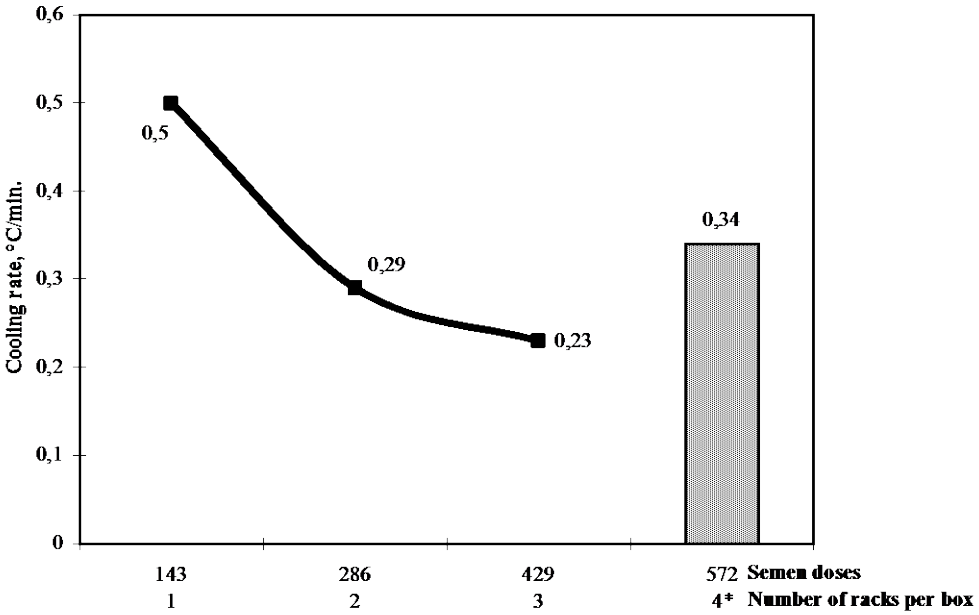

Figure 3. The relationship between semen cooling rate and number of semen doses per box (*Semen was cooled in the open box)

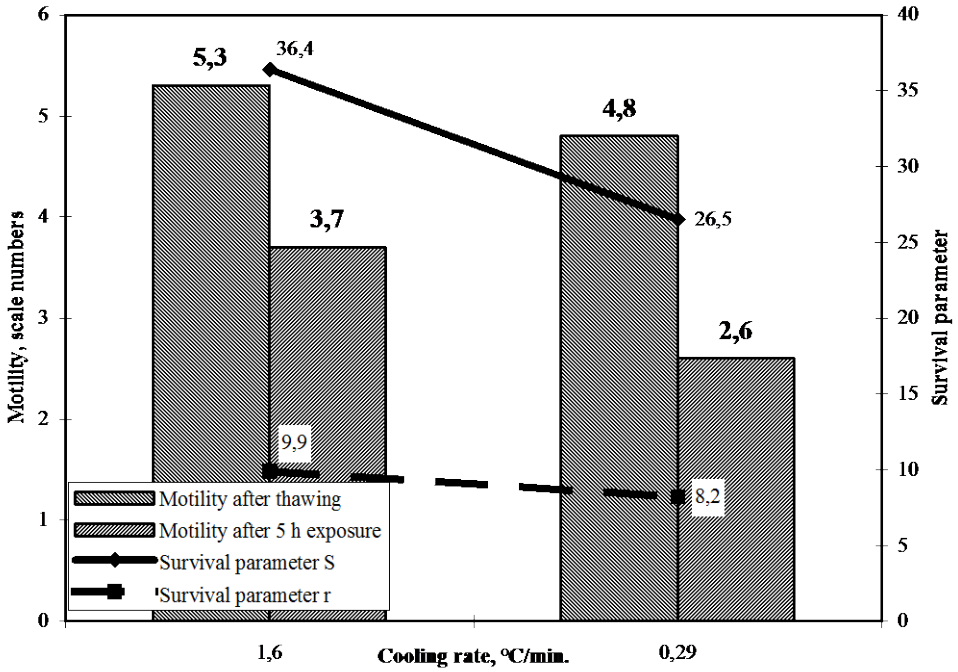

Figure 4. The effect of cooling rate on the physiological parameters of semen

The semen is held in the refrigerator for 180 to 240 minutes (Fig. 5).

The motility of spermatozoa was the highest (5.2±0.1 points) at cooling semen for 240 minutes. This indicator was by 12.6% (P<0.001) and 8.7% (P<0.001) higher if compared, respectively, with semen cooling for 60 and 120 or 180 minutes. The number of spermatozoa with intact acrosomes was the highest for semen cooled for 180 minutes (57.0±0.7%) and

respectively (35.4±0.8) after 5 hour exposure at 38±0.5 °C. The absolute survival indicator (S) of semen cooled for 240 minutes and survival time in hours (r) were, respectively, by 21.7 (P<0.001) and 14.9% (P<0.001) higher in comparison with semen cooled for 180 minutes.

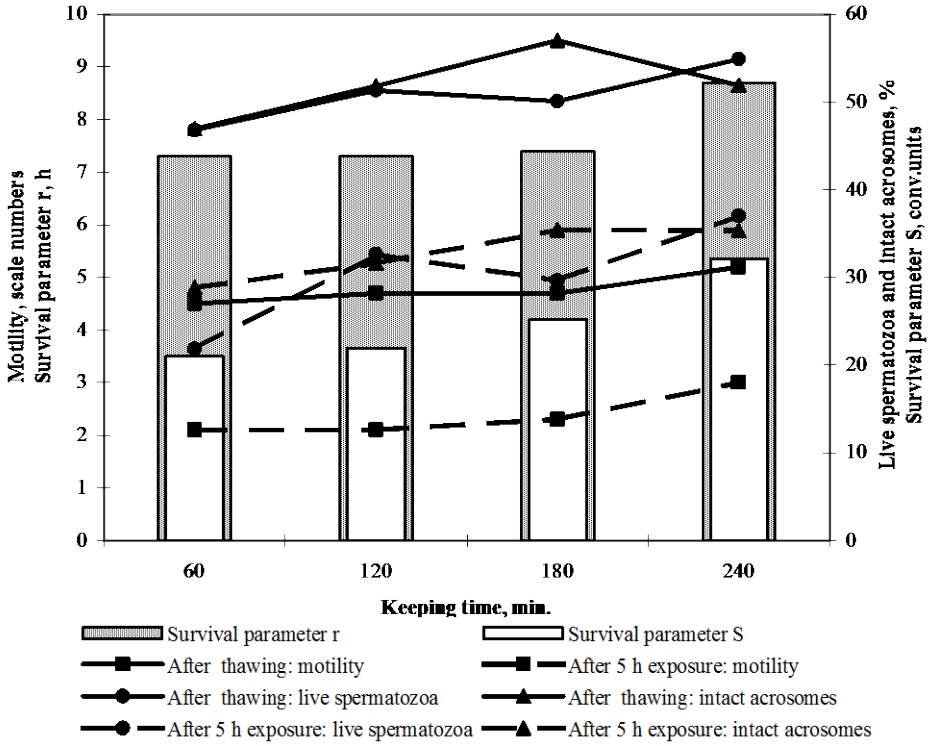

Figure 5. The effect of keeping semen at 4±2 °C on it's quality

Semen freezing

Semen is frozen in biocontainers KV-6202, KhB-0.5 or KhB-0.2 with a special freezing equipment – a copper freezing net. The installation of the safety freezing net in the container KhB-0.5 is carried out without liquid nitrogen present. The freezing equipment for the container KhB-0.2 is fitted on a frame inserted beforehand and afterwards three segments of the freezing net are screwed on it.

If the freezing process is carried out with the gaseous blow, the level of liquid nitrogen in the container should be from 6 to 8 cm below the safety freezing net. The straw racks are taken out of the refrigerator and the straw holders removed quickly. Then the straw racks are put on the safety freezing net.

It is recommended that the temperature 13 mm above the safety freezing net should be maintained at –150±30°C depending on the individual characteristics of bull semen (Fig. 6).

In oder to maintain the stable temperature over the safety freezing net, the determined rate of freezing, and rapid restoring of the temperature after placing of the racks, it is necessary to fill the container with gaseous nitrogen from a gas disposal valve of the storage tanks. The end of the hosepipe should lie on the bottom of the container.

The freezing rate of semen depends on the level of nitrogen under the freezing net or constant temperature maintenance by barbotage with gaseous nitrogen.

The motility of spermatozoa after semen freezing and thawing was the highest (5.5±0.1 points) when semen was frozen at –170±5 °C, and this motility was by 8.6% (P<0.0001) higher in comparison with the control. Absolute survival indicator (S) was by 41.3% (P<0.001) higher when semen was frozen at constant –150±5 °C temperature in comparison with the control.

In the situation when the semen is frozen without blowing the gaseous nitrogen, the safety freezing net should be in touch with liquid nitrogen. The time of freezing is not less

than 6 minutes. The motility of spermatozoa, number of eozinonegative spermatozoa and number of intact acrosomes were, respectively, by 2.0 (P>0.5), 1.3 (P>0.5) and 9.3% (P<0.001) higher of the semen frozen for 4 minutes in comparison with the semen frozen for 2 minutes (Fig. 7). It is suggested to freeze semen for 6 minutes, because in this case the number of spermatozoa with intact acrosomes was the highest both after freezing and thawing and after 5 hour exposure to 38±0.5 °C.

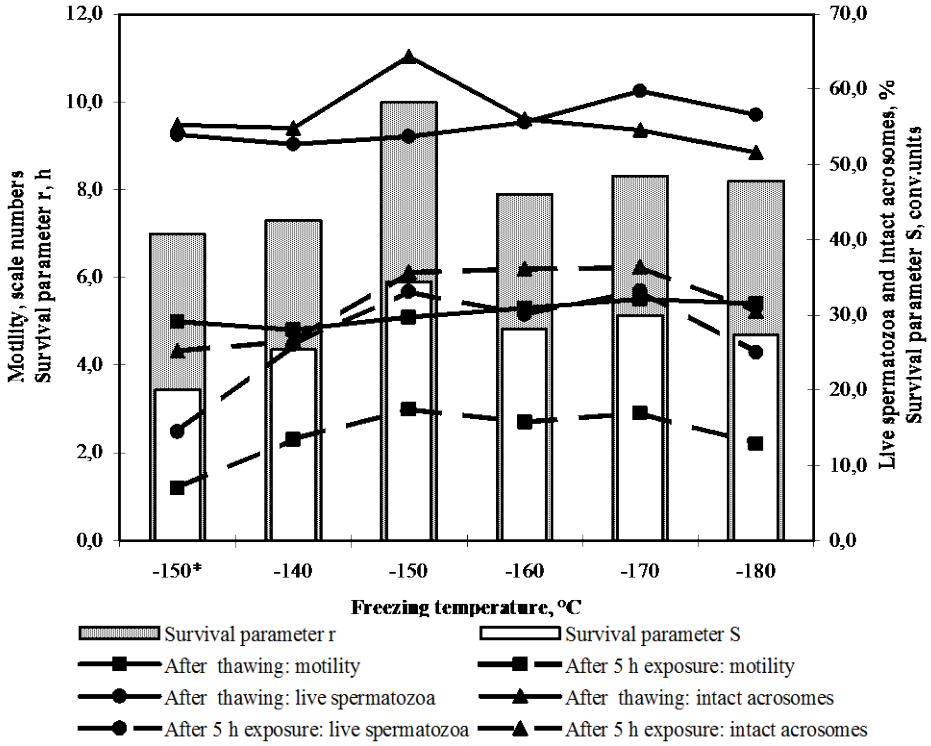

Figure 6. The influence of the freezing temperature on the physiological parameters of semen (*initial temperature)

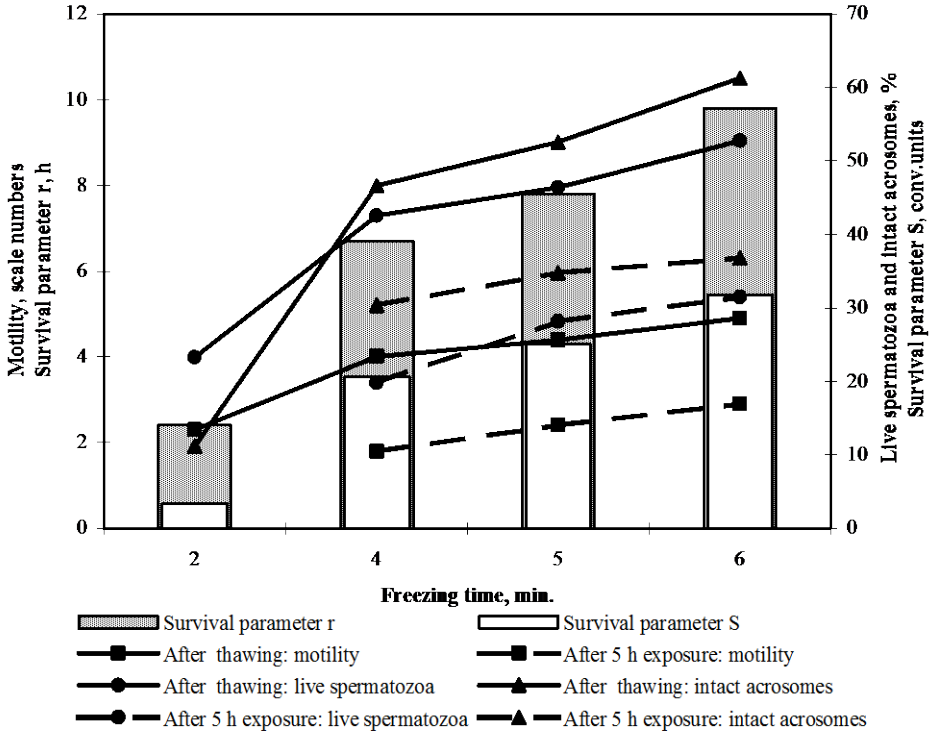

Figure 7. The effect of the freezing time on the physiological parameters of semen (frozen at –150±5 °C)

Absolute survival indicator and survival time were also the highest at freezing semen for 6 minutes. The freezing cycle including temperature stabilization should last for 8 minutes.

The temperature changes of straws are dependent on whether the freezing is carried out with or without barbotage with gaseous nitrogen (Fig. 8). Afterwards, the straws are plunged into big or small plastic goblets filled with liquid nitrogen. It is not allowed to put the straws into empty goblets, because after thawing a part of straws are damaged.

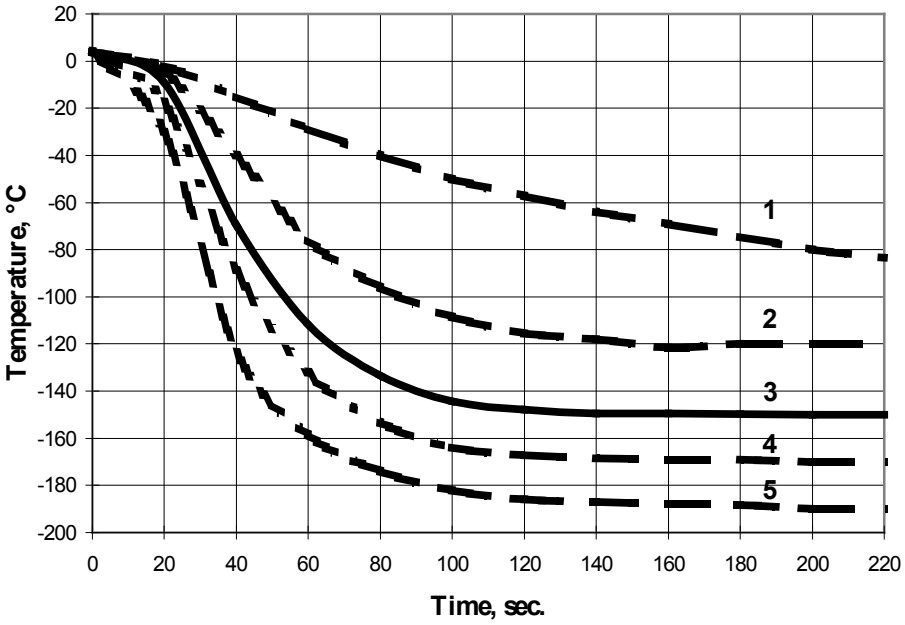

Figure 8. The temperature dynamics of semen frozen in straws using the biocontainer and the freezing net (temperature curve 1 – the lid is used for the stabilization of the ambient temperature; curves 2–5 – the liquid nitrogen barbotage at –120, –150, –170 and –190 °C, respectively, is used for the temperature stabilization at the straw level)

After freezing, semen in goblets is mainly stored in stationary biotanks. The storage of semen necessitates in its presence of liquid nitrogen at the constant temperature of –196 °C. The capacity of a small goblet (height 105 mm, inside diameter 37 mm) is approximately 160 straws. The KhB-0.5-1 type biotank can accommodate 359 canisters with 1795 goblets which store up to 287.000 straws. The capacity of a big goblet (inside height 105 mm, inside diameter 61 mm) is 420 straws. The KhB-0.5-1 biotank can accommodate 144 canisters with 720 goblets, which store up to 302.000 straws.

Thawing of frozen semen

A series of semen collection is understood as the quantity of semen collected from the same bull-donor during one or several mountings, diluted with the same diluent, mixed in the same vessel, frozen during one technological cycle and having the same quality certificate.

In 24–48 hours after freezing the straw is removed from the goblet with a pair of tweezers or special scissors and immediately dipped into a water bath controlled automatically at 40±0.5 °C. Semen thawing takes 10 seconds.

After thawing, the semen should meet the following minimum standards (Table 3).

Table 3. Minimum requirements for the quality and veterinary standards of thawed semen

Item	Requirement
Colour and appearance	Uniform liquid mass of mat yellowish grey to yellow colour (depending on the diluent used)
Sperm motility, no less than (scale numbers; %)	4; 40
Progressive movement of sperm in a dose, no less than (million)	15.0
Absolute survival rate, no less than (conv. units)	12
Sperm livability at +38±0.5°C, no less than (h)	5
Sperm fertility rate, no less than (%)	50
Coli titre	Negative
Pathogenic microorganisms	Should not be detected

On agreement with the customer, the producer may prepare semen with the sperm motility lower than 4 scale numbers (40%) and the number of motile spermatozoa per dose lower than 15 million.

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