Praca oryginalna *Original paper*

Determination of optimum stallion semen freezing regimes

JONAS KUTRA, VIDMANTAS PILECKAS, ALGIRDAS URBSYS, ARTORAS SIUKSCIUS, RASA NAINIENE

Institute of Animal Science of the Lithuanian Veterinary Academy,
R. Zebenkos 12, LT-82317 Baisogala, Radviliskio r., Lithuania

Kutra J., Pileckas V., Urbsys A., Siukscius A., Nainiene R.

Determination of optimum stallion semen freezing regimes

Summary

Semen cryopreservation opens new possibilities for the development of breeding work and preservation of the gene pool in horse breeding. Currently a relatively small number of mares are being inseminated with frozen semen. Therefore, the improvement of semen deep-freezing and thawing regimes is important for further studies of stallion semen cryopreservation processes.

The methods of animal semen freezing on dry ice and in convectional liquid nitrogen vapor stream are carried out by simple technical means, and, what is more, large quantities of semen can be frozen at a time. However, programmed equipment involving a larger input allows the implementation and reproduction of desirable freezing regimes more accurately and to extend the range of their parameters.

The semen was frozen in two ways: in convectional liquid nitrogen vapor stream on a metal perforated shield, fitted in biostorage KS-40 equipment and in Minicool AS-25. Three freezing regimes characterized by the super cooling temperature of the free water in the semen were realized using the Minicool AS-25 equipment. The efficiency of the freezing regimes was determined by post-thaw motility of spermatozoa.

The results from the study indicated that the quality of frozen semen of most stallions was invariable regardless of freezing methods and regimes. Thus, semen can be frozen by convection flow of nitrogen vapor using common equipment and lower amounts of freezing agent.

Optimum freezing regimes should be sought for cryopreservation of stallion semen known as „unstable freezing". These regimes could be ensured by using the equipment suited to the reproduction of the identical freezing regime for every specific stallion's semen. During the experiment there was only one stallion identified as having an optimum semen freezing regime, yet the experiment showed that in order to reach optimum conditions, a freezing parameter changing gradient could be determined in stallion semen cryopreservation. In the latter case the parameter characterizing freezing regimes could be the super freezing temperature of the free water in the semen and the optimum criterion could be post-thaw motility of spermatozoa.

Keywords: stallion, semen

Semen cryopreservation opens new possibilities for the development of breeding work and preservation of the gene pool in horse breeding. At present, a relatively small number of mares are being inseminated with frozen semen. Therefore, the improvement of the operating conditions for semen deep-freezing and thawing is important for further studies of stallion semen cryopreservation processes (10). Ivanov (4) was the first to discover that stallion spermatozoa retained post-thaw motility after freezing at –15°C but were lost at –100°C. Since 1964, rapid animal semen freezing techniques on dry ice or in liquid nitrogen vapor have been developed (5). Simple technical means are applied and, moreover, large quantities of semen can be frozen at a time. However, our observations in practice indicated that stallion semen freezing in convectional liquid nitrogen vapor stream resulted in different freezing results. Even the semen freezing results of one and the same stallion were different – sometimes better, sometimes worse.

Until now there are disagreements as to the most favorable method or operating regime conditions for stallion semen cryopreservation. It is suggested to freeze the semen packaged into 0.5 cm^3 straws in convectional liquid nitrogen vapor stream by keeping the temperature from –120 to 160°C or to freeze at a rate of –60°C/min (11). Freezing of semen packaged into 0.25 cm^3 straws in liquid nitrogen vapor medium, the temperature of which is kept from –120 to –140°C, should involve a different exposition time that is unrelated with the parameter characterizing the regime in use (7).

Programmed equipment can also be used for semen freezing, and that allows the implementation and to reproduce the desirable freezing regimes more accurately and to extend the range of regime parameters. At the

start (from 37 to 5°C) semen should be cooled at a rate of 0.7°C/min, later from 0.05 to 0.5°C/min. Post-thaw motility of spermatozoa is usually an indicator of the quality of frozen semen, efficiency of the freezing method, and expediency of the freezing regime (8, 9).

The purpose of the study was to investigate the main presumptions for the determination of the optimum stallion semen freezing methods and operating conditions.

Material and methods

The study was carried out at the joint-stock company „Vilniaus zirgynas" and the LVA Institute of Animal Science. Conventional housing and feeding were applied to the stallions the semen of which had been studied. Fresh stallion semen met minimal requirements for ejaculation volume – no less than 10 cm^3, total spermatozoa count per ejaculation – no less than 1.5 milliard, density – not lower than 0.15 milliard/cm^3 and sperm motility – not lower than 60%.

The composition of the extender used for semen cryopreservation is presented in tab. 1.

Tab. 1. Extender composition used for stallion semen freezing

Ingredients	Quantity
Redistilled water, cm^3	100
Lactose, g	11.0
Sodium citrate, g	0.008
Egg yolk, cm^3	10
Glycerol, cm^3	3

Fresh semen was diluted with 27 ± 1°C extender at a rate of 1 : 1, stored at a temperature of 19 ± 1°C for 15 min and afterwards centrifuged at the 680-1200 rcf. The relative centrifugal force (rcf, g-force) was calculated according to the following formula:

$$rcf = 1.118 - 10^5 - n^2 - r,$$

where n – rotation speed in 1/min;
r – centrifuging radius in cm (r = 6.75 cm in our case).

The concentrated semen was diluted so as to have no less than 150 million motile spermatozoa per insemination dose (it is assumed that spermatozoa motility after thawing will be no less than 25%). The diluted semen was packaged into straws to be spread in one layer with an interstice into cassettes. The cassettes were then transferred into the fridge and cooled at 4 ± 2°C temperature for 2.5 h.

The semen was frozen in two ways: in liquid nitrogen vapor on a metal perforated shield fitted in the biostorage KS-40 and in Minicool AS-25 equipment. The height of the shield plane above liquid nitrogen surface can be changed to regulate the temperature of liquid nitrogen vapor stream before freezing. In our experiments, the height of the shield before freezing was fixed to have a temperature of –135 ± 5°C at the level of straws laid on the shield. This method of freezing ensures temperature changes inside the straws in the range indicated in fig. 1. The exact temperature change regime in each case depends on many factors: on the number of semen doses frozen simultaneously, on the position of the straw in connection with the shield perimeter, on the ambient conditions (air temperature, relative humidity), on the intensity of liquid nitrogen vapor convection stream, etc. This way of freezing does not realize an identical semen temperature changing regime in each case.

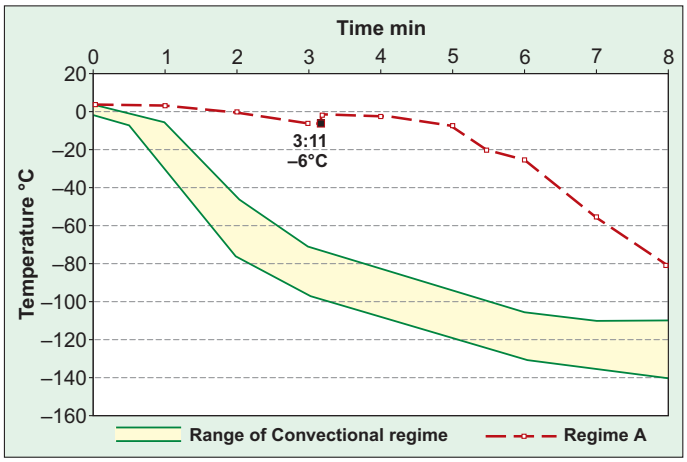

Fig. 1. Possible range of semen temperature changes at convectional freezing; for comparison: realized freezing regime using Minicool AS-25 (regime A; characteristic parameter – superfreezing temperature of free water –6°C)

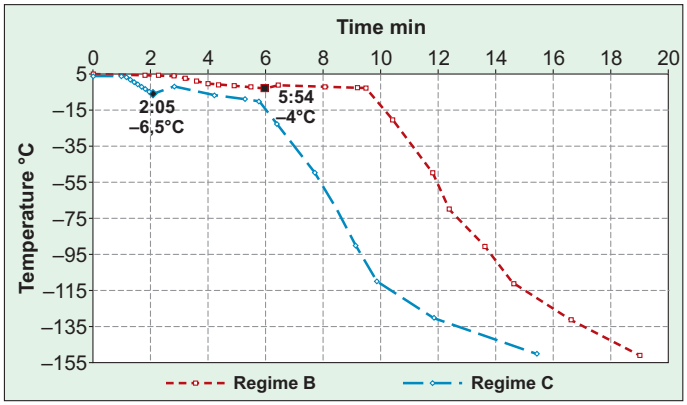

Fig. 2. Freezing regimes realized with Minicool AS-25 (B – characteristic parameter – –4°C and C – –6°C)

The freezing regime in Minicool AS-25 is realized in a closed chamber in the environment of the turbulent vapor stream of liquid nitrogen with a dynamic 1.5% error of the temperature maintenance. In our experiments, Minicool AS-25 was used to carry out three freezing regimes (regimes A, B and C; fig. 1 and 2) that are best characterized by free water superfreezing temperature in the freezing semen.

After freezing, straws with semen were immersed into liquid nitrogen and kept there no less than 3 days before thawing.

The efficiency of the freezing regime was determined by post-thaw motility of spermatozoa. The semen in the straw was thawed in 10 s in a 38 ± 0.5°C water bath. Sperm motility was visually evaluated with the biologic microscope by determining the ratio between the number of motile spermatozoa and total spermatozoa number in three 100 × microscopic fields of each sample.

It is assumed that after thawing semen is suitable for insemination provided the number of motile spermatozoa accounts for no less than 20%.

The results of motility evaluation are expressed in mean values with standard deviation (M + SD). The differences in the efficiency of the freezing regimes were estimated after the analysis of the post-thaw sperm motility data using Microsoft Office Excel 2003 ANOVA single factor analysis tool. Differences were considered significant at $P < 0.05$.

Results and discussion

One part of semen collected from four sires was frozen by the convectional method, another part by Minicool AS-25 regime A. Post-thaw sperm motility results are presented in fig. 3.

The ANOVA indicated that at this stage of the experiments the quality of frozen semen (post-thaw motility of spermatozoa) did not depend on the freezing method applied and that possible differences could be the result of individual traits of the stallions. By this indicator, the semen of the stallions Kaukas and Agentas was suitable for insemination, irrespectively of the freezing method, and the semen of Koralas was found to be unsuitable. However, sperm motility of the stallion Kosmosas was determined to be twice as high when the semen was frozen by the convectional method (25.6 ± 1.71%) compared with Minicool AS-25 regime A freezing (12.5 ± 2.59%). Thus it can be presumed that semen quality of individual stallions (in the latter case, Kosmosas) may depend on the applied freezing method or, to be more specific, on the semen temperature changing regime that is best characterized by the free water superfreezing temperature in the semen.

In order to check this presumption, in the next stage of the experiment four stallions were chosen again for semen cryopreservation studies. These stallions (including Kosmosas) were chosen because their semen frozen by the convectional method was subjectively evaluated as having „unstable freezing" characteristics in the period of observation that lasted from 3 to 12 months.

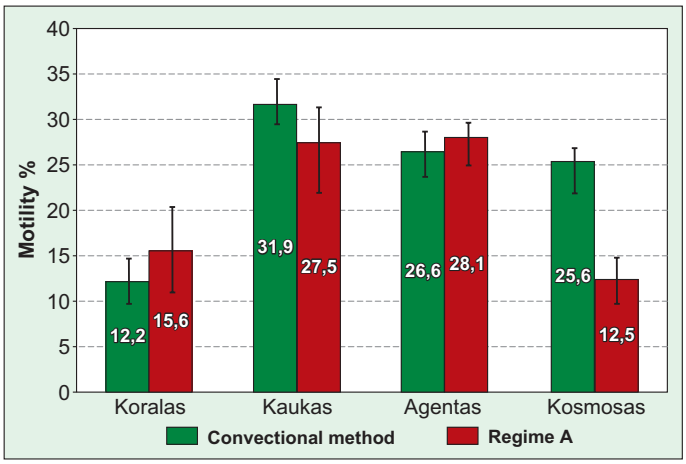

Fig. 3. Post-thaw motility of spermatozoa frozen using different methods (n = 16)

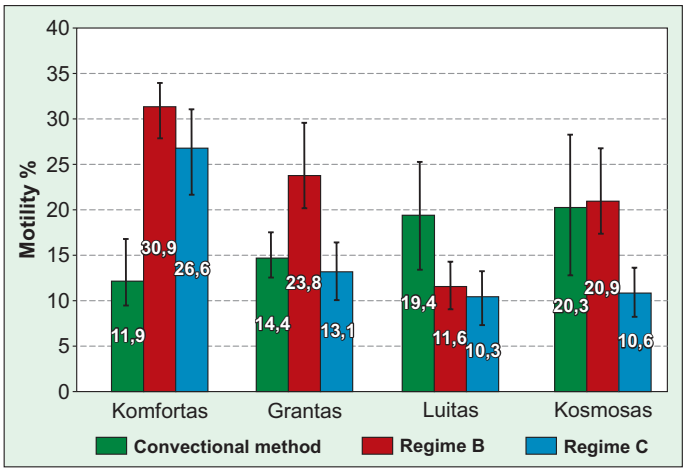

Fig. 4. Post-thaw motility of spermatozoa cryopreserved in three different methods (n = 16)

At this experimental stage, stallion semen, divided into three parts, was frozen using different freezing regimes, i.e. using a convectional method and two limiting regimes B and C that could be realized with Minicool AS-25.

The experiment indicated that the convectional freezing method was most suitable for Luitas semen. This method of conservation was also suitable for Kosmosas semen. However, freezing regime B was found to be more favorable, also for stallions Komfortas and Grantas. The least favorable semen freezing method for most stallions (except Komfortas) was regime C.

The ANOVA indicated that post-thaw motility of spermatozoa depended both on the individual traits of the stallion and on the freezing method applied (fig. 4). Optimum semen freezing regime (regime B) was identified only for one stallion, Grantas; however, it is evident that changing the gradient for freezing regime parameters can be determined for each stallion semen cryopreservation applied at this stage.

Conclusions

1. The frozen semen quality of most stallions is invariable as regards freezing methods and regimes. Thus, the semen can be frozen by the convectional method using ordinary equipment and having a lower expenditure of freezing agent.

2. The optimum freezing regime should be found for cryopreservation for the semen that is characterized as having „unstable freezing". In this case, equipment that can reproduce an identical freezing regime for each individual stallion semen should be used.

3. Superfreezing temperature of the free water present in the semen can be the parameter characterizing the freezing regime, while the number of motile spermatozoa after thawing can become the optimum criteria.

References

1. *Bedford S. I., Graham J. K., Amann R. P., Squires E. L., Picket B. W.*: Use of two freezing extenders to cool stallion spermatozoa to 5°C with and without seminal plazma. Theriogenology 1995, 43, 939-953.
2. *Blottner S., Warnke C., Tuchscherer A., Heinen V., Torner H.*: Morphological and functional changes of stallion spermatozoa after cryopreservation during breeding and non-breeding season. Anim. Reprod. Sci. 2001, 65, 75-88.
3. *Crockett E. G., Graham J. K., Bruemmer J. E., Squires E. L.*: Effect of cooling of equine spermatozoa before freezing on post-thaw motility: Preliminary results. Theriogenology 2001, 55, 793-803.
4. *Ivanov I. I.*: Izbrannye trudy. Moscow: Kolos 1970, 319-321.
5. *Jondet R.*: Congelotion rapide du sperme de taureau conditionne en pallettes. Vth Congr. Anim. Reprod., Trento 1964, 4, 463-467.
6. *Jondet R.*: Meintier du pouvair fecondant du sperme de taureau conditionne on paillettes, apres congelation rapide dans les vapeurs d'azota ligreide. Bull Acad. Vet. France 1964, 37, 333-343.
7. *Kurbatov A. D., Platov E. M., Korban R. V.*: Kriokonservacija spermy sel'skohozjajstvennyh zhivotnyh. Moscow: Agropromizdat 1988, 78-79, 86, 92, 94, 255.
8. *Leipold S. D., Graham J. K., Squires J. R., McCue P. M., Bunsko S. P., Vandewol D. R.*: Effect of spermatozoal concentration and number on fertility of frozen equine semen. Theriogenology 1998, 49, 1537-1543.
9. *Samper J. C.*: Management and fertility of mares bred with frozen semen. Anim. Reprod. Sci. 2001, 68, 219-228.
10. *Squires E. L.*: Integration of future biotechnologies into equine industry. Anim. Reprod. Sci. 2005, 89, 187-198.
11. *Vidament M.*: French field results (1985-2005) on factors affecting fertilities of frozen stallion semen. Anim. Reprod. Sci. 2005, 89, 115-136.

Author's address: PhD Jonas Kutra, R. Zebenkos 12, LT-82317 Baisogala, Radviliskio r., Lithuania; e-mail: reprodukcija@lgi.lt