COMPARATIVE EVALUATION OF BOVINE SEMEN CRYOPRESERVATION METHODS AND EXTENDERS

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Abstract. The purpose this study was to carry out a comparative evaluation of semen cryopreservation methods and extenders. The study indicated that in thawed pellets sperm motility was by 40.7 and 88.9 % ($P<0.001$) lower and absolute survival by 11.9 and 77.8 % lower in comparison with the semen frozen in 0.25 ml straws by, respectively, Baisogala and Lithuanian technologies. Duration of sperm survival was 5.8 % higher in pellets than in straws frozen by the Baisogala technology, but it was 32.7 % lower if compared with the Lithuanian cryopreservation technology ($P<0.01$). Spermatozoa survival rate was 12.1 and 26.3 % higher in semen frozen by Baisogala and Lithuanian technologies in comparison with semen freezing in pellets ($P<0.05$ and $P<0.01$).

A comparative evaluation of the extenders used for semen freezing indicated that pot-thaw sperm motility in the semen frozen with LGCK (lactose-glycerol-citrate egg yolk) control extender by the Lithuanian technology was 4.5 % higher than that in the semen frozen using Biociphos Plus extender but 6.8 % lower in comparison with Bioxcell extender usage. However, semen freezing with Biociphos plus and Bioxcell extenders resulted in insignificant differences regarding sperm motility and survival rate. The study indicated that absolute survival was 19.2 % higher and spermatozoa survival time was 1.2 hour longer when LGCK (lactose-glycerol-citrate-egg yolk) extender was used compared with Bioxcell usage ($P<0.01$ and $P<0.001$). It is recommended to use LGCK or Bioxcell extenders when bovine semen is preserved by the Lithuanian technology.

Keywords: bovine semen, extenders, cryopreservation

Introduction. It is impossible to control semen quality at animal mating; always there is a risk of spreading infectious, fungous and protozoa diseases. Semen freezing is the only sufficiently complete method of semen collection that allows to preserve the genetic code for an indefinite time, yet the power of fecundation by cryopreserved semen is lower than that of fresh one (Watson P., 2000). In 1936, disposable straws were suggested for semen packaging. The suggestion was worked out by Sorensen E. (1960) in Denmark and afterwards improved and developed by Cassou R. (1964) in France. Later, Minitube semen cryopreservation technique was developed in Germany. In Lithnuania, Pakėnas P. developed Lithuanian technology of housing and usage of breeding bulls (1983) which was used by approximately 40 breading enterprises in the Soviet Union (Pileckas V., 2002). The quality of frozen semen depends on sire feeding, frequency of semen collection (Pakėnas P., 1993), technological measures used for semen preparation, and semen thawing temperature (Ahmad Z. et al., 2003; Alm K. et al., 2002). One of the most important tasks at semen cryopreservation was to prepare extenders that not only increased semen volume but also protected spermatozoa from negative factors during semen dilution, cooling and freezing-thawing periods (Karabinus D. S. et al., 1991). Low density lipoproteins of egg yolk and milk powder used in extenders might destabilize spermatozoa membranes (Bergeron A., et al., 2006) and, beside, their usage might influence the qualitative parameters of extenders and increase the risk of infectious diseases (Bousseau S. et al., 1998). The role of extenders is to protected spermatozoa from cold shock and agglutination, maintain stable pH value and osmotic pressure (Foote R. et al., 1987; Garner D. et al., 2001; Chen Y. et al., 1993). The composition of many commercial extenders such as Laicipfos 478, Biociphos PLUS, TRYLADIL Konzentrat, BULLXcell is not revealed, they are presented to the consumer only by indicating the methods of their preparation. The purpose of our study was to determine the effects of different extenders on bovine spermatozoa when semen is frozen either in straws or in pellets.

Materials and methods. The study was carried out at the Animal Reproduction Department of the LUHS (Lithuanian University of Health Sciences), Institute of Animal Science and Marijampole Department of the joint-stock company "Lietuvos veislininkystė"

Semen was collected twice a week with two ejaculates at a time by artificial vagina. In this study, semen with sperm motility not lower than 7 points (70 %) and concentration not lower than 0.8 milliard/ml was used.

Semen motility was determined in fresh semen and after semen freezing-thawing by optical microscope NICON ECLIPSE E200 with electric 38±1° c heating plate and using the Sperm Class Analyser for evaluation of semen qualitative parameters. Semen was thawed in water for 9 seconds in a 38±1° C water bath with following motility evaluation. Semen motility was also evaluated following prolonged incubation of thawed semen in the water bath at 38±° C for 5 hours (Pakenas P., 1993).

The temperature during semen freezing in storage places, freezing device and straws were controlled by

electronic thermometer Recorder KD7 (produced – Lumel, Poland).

Spermatozoa survival was determined by the dyeing method after semen thawing using water soluble eosin dyes prepared by the methods of Bjorndahl L. et al. (2003).

When the semen was frozen in uncoated pellets (control) on foam plate, it was diluted after semen collection and evaluation using 32±1° C extender made from lactose 11.5 g, glycerol 5 ml, egg yolk 20 ml and distilled water 100 ml (Nagase H. et al., 1964) at a rate of no less than 15 million motile spermatozoa per 0.5 ml dose after semen thawing. The diluted semen was cooled at 2–5° C for 3 to 4 hours and afterwards frozen on a 25×25×2 cm foam plate with 0.5 ml holes. The semen was thawed in 2.9 % sodium citrate solution at 38±1° C.

When the semen was frozen by Baisogala technology, mixed sperm ejaculates were used in the study. The semen was diluted using the extender composed of lactose 11.5 g, glycerol 5 ml, egg yolk 20 ml and distilled water 100 ml. After quality evaluation, the semen was diluted with 32±0.5° C extender by pouring 5 ml extender into each semen collector. After mixing, semen was diluted to reach the final concentration of no less than 15 million motile spermatozoa per semen dose after packaging. The diluted semen was cooled for 20–25 minutes at a thermostat at 22±0.5° C. The semen was then packaged into 0.25 ml straws and stored for 4 hours in the refrigerator at 4±2° C. Afterwards, straws were frozen for 5 minutes in a nitrogen container on shield copper at -120±5° C. After semen freezing, the straws were plunged into liquid nitrogen. The semen was thawed for 9 seconds in a 38±1° C water bath.

Mixed sperm ejaculates were used when the semen was frozen by the Lithuanian technology. The semen was diluted using the extender composed of lactose 11.5 g, glycerol 5 ml, egg yolk 20 ml and distilled water 100 ml. After quality evaluation, semen was diluted at a rate 1:1 with 27±1° C extender and kept for 15 minutes at 19±1° C. To reach the required concentration, the semen was diluted for the second time with 19±1° C temperature extender. After packing into 0.25 ml straws the semen was stored in as refrigerator at 4±2° C for 4 hours. After cooling, the straws were frozen for 6 minutes in a nitrogen container on a copper shield at -150°±0.5 C and plunged into liquid nitrogen. The semen was thawed for 10 seconds in a 40±1° C water bath.

In order to determine the effects of the extenders on sperm motility, viability and sperm survival time, bovine semen was frozen according to the Lithuanian technology. The ejaculates from the same bull divided into four parts were used. The semen was diluted using a modified extender composed of lactase 10.5 g, glycerol 5 ml, sodium citrate 0.2 g, egg yolk 20 ml and distilled water 100 ml (LGCK) (control). Besides, Bioxcell and Biociphos plus extenders were used.

Sperm survival (percentage of live spermatozoa) was determined subjectively in microscope and following sperm dyeing with eosin solution.

The coefficient kj of spermatozoa motility decline was determined after semen thawing and 5 hours incubation in a 38±0.5° C water manufacturer of the bath. This coefficient was expressed as the ratio between sperm motility after 5 hours incubation at 38±0.5° C and sperm motility after thawing. The closer was the ratio to 1 the better was the semen quality. Sperm survival time in hours is the period from the trial start till the moment when spermatozoa lose the ability to move straight-forward by excluding half of the period between the next-to- last and the last study of spermatozoa motility (Pakėnas P. 1993).

Absolute survival (S) is the indicator of spermatozoa survival determined by the methods of Kurbatov A. D. (1988).

The data were processed using statistical package R, version 2.0.1 (Gentlemen, Ihaka, 1997). The significance of the differences was determined by Student and considered significant at P<0,05.

Results. The comparative evaluation of bovine semen cryopreservation technologies indicated that sperm motility in thawed pellets was 40.7 and 88.9 % (P<0.001) lower compared with the semen frozen in 0.25 ml straws by Baisogala and Lithuanian technologies. However, after 5 hours incubation at 38±0.5° C, sperm motility was 25 % (P<0.01) higher when frozen in pellets that frozen by the Baisogala technology, yet 69.9 % (0<0.01) lower in comparison with semen freezing by the Lithuanian technology (Table 1).

Absolute survival rate of semen frozen in pellets was 12.0 and 77.8 % lower than that of semen frozen in straws by Baisogala and Lithuanian technologies. Sperm survival time was 5.8 % higher when freezing semen in pellets than in straws by the Baisogala technology, but 32.7 % lower in comparison with the Lithuanian technology (P<0.01). Sperm motility decline coefficient (kj) was 57. 1 % higher for the semen frozen in pellets than for the semen frozen in straws by the Baisogala technology (P<0.001), but 67.1 % lower than that for the semen frozen by the Lithuanian technology (P<0.01). The percentage of live spermatozoa in the semen frozen by Baisogala and Lithuanian technologies was, respectively, by 41.2 and 89.5 (P<0.001) % higher than that of the sperm in pellets.

Comparative evaluation of extenders for bovine semen freezing indicated that post-thaw spermatozoa motility under the Lithuanian technology with LGCK(lactose-glycerol-citrate-egg yolk) (control) extender was 4.5 % higher than that with Biociphos plus, but 6.8 % lower with Bioxcell extender usage for semen freezing. The time of freezing is not less than 6 minutes (Table 2).

However, the differences of spermatozoa motility using the above extenders were insignificant. After thawing, sperm incubation for 5 hours at 38±0.5° C resulted in 64.3 % (P<0.001) higher spermatozoa motility when LGCK (lactose-glycerol-citrate-egg yolk) extender was used in comparison with Biociphos plus, but his indicator was 58.3 % lower with Bioxcell extender usage for semen freezing.

Table 1. **Comparative evaluation of bovine semen cryopreservation technologies** (n=20)

Technology	Post-thaw motility of spermatozoa in scale number	Motility of spermatozoa after 5 h exposure at 38 ± 0.5°C	Absolute survival rate (S)	Survival time (h)	Decline in sperm motility coefficient kj	percentage of live spermatozoa
Pellets (control)	2.7±0.09	0.8±0.19	11.7±0.67	5.2±0.26	0.28	29.4±2.8
Baisogala	3.8±0.07	0.6±0.16	13.1±0.54	4.9±0.18	0.16	41.5±1.9
Lithuanian	5.1±1.0	2.3±1.3	20.8±0.1	6.9±0.2	0.47	55.7±3.7

Table 2. **Effect of freezing extenders on sperm motility, survival and duration of survival** (n =10)

Extender	Post-thaw sper motility (points)	Motility of spermatozoa after 5 h exposure at 38 ± 0.5°C	Absolute survival rate (S)	Survival time (h)	percentage of live spermatozoa
LGCK (control)	4.4±0.1	1.4±0.1	21.9±0.5	6.7±0.1	55.7±4.3
Biociphos plius	4.2±0.1	0.5±0.2	17.7±0.8	5.6±0.3	58.0±0.6
Bioxcell	4.7±0.1	2.4±0.1	31.2±0.6	7.4±0.2	58.5±1.5

The study indicated that absolute survival rate was 19.2 % higher in the semen frozen with LGCK extender that with Biociphos plus extender, but was 42.5 % lower when the semen was frozen using Bioxcell extender ($P<0.001$). The length of spermatozoa survival time in post-thaw semen was 6.7±0.1 hours, when the semen was frozen with LGCK extender and that was 1.2 hour longer compared with Biosiphos plus extender ($P<0.01$) and 1.4 hour shorter compared with Bioxcell extender usage .

There were no significant differences for spermatozoa viability in post-thaw semen found using the above extenders.

Discussion and conclusions. Post-thaw spermatozoa motility in pellets was lower than that when the semen was frozen in 0.25 ml straws following Baisogala and Lithuanian technologies ($P<0.01$ and $P<0.001$). However, after 5 hours incubation at 38±0.5° C, spermatozoa motility in pellets was higher ($P<0.01$) than that in Baisogala technology straws, but lower than in Lithuanian technologies. Award M. M. et al (2004) used LGK (lactose-glycerol-egg yolk) extender for semen freezing and found that post-thaw spermatozoa motility was 47 % higher when semen was frozen in 0.25 ml straws than in pellets, Al-Badry K, I. (2012) used 0.25 ml straws for semen freezing and reported that post-thaw spermatozoa motility was by 19.4 % lower.

After semen thawing, no significant differences were found for spermatozoa motility and survival when LGCK, Biociphos plus and Bioxcell extenders were used for semen freezing. According to Amirad L. et al (2005), using Biociphos extender resulted in 64.1±17 % post-thaw spermatozoa motility. The use of extenders without animal products reduces the risk of infectious disease spreading and allows to freeze the semen of not only domestic (Gil J. et al., 2000; Van–Wagtendonk–de Leeuw A. et al., 2000; Fukui Y. et al., 2008) but also of wild animals (Perez-Garnelo S. S. et al., 2006; Herold F. S., 2004). However, the effect of such extenders on semen physiological parameters is different as indicated in literary sources (Januskauskas A. et al., 2003; Leite T. G. et al., 2010). Tubassoni M. et al. (2013) reported 40.5±1.7 % spermatozoa motility and 60.0±1.5 % spermatozoa survival, whereas Khaki A. et al (2013) reported 40.7±1.8 % spermatozoa motility and 60.8±1.4 % survival when TRIS, glycerol, egg yolk extender was used for semen freezing. Arifiantini R. I. et al (2010) found no significant differences for spermatozoa motility and survival when TSM, Andromed and TEY extenders were used for semen freezing. According to Moce E. et al (2006), post-thaw spermatozoa motility and survival were, respectively, 17 and 35 % when the freezing extender contained 20 % egg yolk and 8.75 % glycerol. Nova-Trujillo H. et al (2011) found 33.9±12.4 % spermatozoa motility and 500±14.1 % survival when the semen was frozen with the extender containing 20 % egg yolk and 7 % glycerol and the diluted semen was cooled at 4° C for 4 hours and after packing frozen at -120°±0.5 C for 10 minutes. It is recommended to used LGCK or Bioxcell freezing extenders for bovine semen cryopreservation by the Lithuanian technology.

References

1. Ahmad Z., Anzar M., Shahab M. et al. Sephadex and sephadex ion exchange filtration improves the quality and freezability of low–grade buffalo semen ejaculates. *Theriogenology.* 2003. 59. P. 1189–1202.

2. Al-Badry K. I. Effect of Various Thawing Times and Temperatures on Frozen Semen Quality of Friesian Bulls in Iraq. *International Journal of Animal and Veterinary Advances.* 2012. 4(6). P. 384–388.

3. Alm K., Dahlbom M., Saynäjarvi M. et al. Impaired semen quality of AI bulls fed with moldy hay: a case report. *Theriogenology*. 2002. 58. P. 1497–1502.

4. Amirat L., Anton M., Tainturier D., Chatagnon G., Battut I., Courtens J. L. Modifications of bull spermatozoa induced by three extenders: Biociphos, low density lipoprotein and Triladyl, before, during and after freezing and thawing. *Reproduction*. 2005. 129. P. 535–543.

5. Arifiantini R. I., Yusuf T. L. Developing of Tris Soy Milk Diluent for Frisian Holstein Bull Frozen Semen. *Journal of Biosciences.* 2010. 17(2). P. 91–94.

6. Awad M. M., Graham J. K. A new pellet technique for cryopreserving ram and bull spermatozoa using the cold surface of cattle fat. *Animal Reproduction Science.* 2004. 84(1). P. 83–92.

7. Bergeron A., Manjunath P. New insights towards understanding the mechanisms of sperm protection by egg yolk and milk, *Molecular Reproduction and Development.* 2006. 73. P. 1338–1344.

8. Bousseau S., Brillard J., Marquant–de Guienne B. Comparison of bacteriological qualities of various egg yolk sources and the in vitro and in vivo fertilizing potential of bovine semen frozen in egg yolk or lecithin based diluents. *Theriogenology*. 1998. 50. P. 699–706.

9. Cassou R. La methode des paillettes en plastique et son adaption la generalization de la congelation. *Comptes Rendus de l'Academie d'Agriculturie de France*. 1964. No. 50. P. 881–887.

10. Chen Y., Foote R., Tobback C. Survival of bull spermatozoa seeded and frozen of different rates in egg yolk–tris and whole milk extenders. *Journal Dairy Science*. 1993. 76. P. 1028–1034.

11. Foote R., Arrióla J. Motility and fertility of bull sperm frozen-thawed differently in egg yolk and milk extenders containing detergent. *Journal Dairy Science.* 1987. 70. P. 2642–2647.

12. Fukui Y., Kohno H., Togari T., Hiwasa M., Okabe K. Fertility after artificial insemination using a soybean-based semen extender in sheep. *Journal of Reproduction and Development*. 2008. 54. P. 286–289.

13. Garner D., Thomas A., Gravance C. Seminal plasma addition attenuates the dilution effect in bovine sperm. *Theriogenology.* 2001. 56. P. 31–40.

14. Gil J., Januskauskas A., Håård MCh. Functional sperm parameters and fertility of bullsemen extended in Biociphos–Plus and Triladyl. *Reproduction of Domestic Animal.* 2000. 35. P.69–77.

15. Herold F.S., Aurich J.E., Gerber D. Epididymal sperm from the African buffalo (*Syncerus caffer*) can be frozen successfully with Andromed and with Triladyl but the addition of bovine seminal plasma is detrimental, *Theriogenology*. 2004. 61. P. 715–724.

16. Januskauskas A., Johannisson A., Rodriguez-Martinez H., Subtle membrane changes in cryopreserved bull semen in relation with sperm viability, chromatin structure, and field fertility. *Theriogenology.* 2003.60. P. 743–758.

17. Karabinus D.S., Evenson D.P., Kaproth M.T. Effects of egg yolk–citrate and milk extenders on chromatin structure and viability of cryopreserved bull sperm. *Journal Dairy Science*. 1991. 74. P. 3836–3848.

18. Khaki A., Batavani R. A., Najafi G. The *in vitro* effect of leptin on semen quality of water buffalo (*Bubalus bubalis*) bulls. *Veterinary Research Forum.* 2013. 4(1). P. 7–12.

19. Kurbatov A. D., Platov J. M., Korban R. V. et al.,Kriokonservacija spermy selskochoziajstvennich zivotnich. Agropromizdat. 1988. P. 86–87

20. Moce E., Graham J. K. Cholesterol-loaded cyclodextrins added to fresh bull ejaculates improve sperm cryosurvival. *Journal of Animal Science.* 2006. 84. P. 826–833.

21. Nagase H., Niwa T. Deep freezing bull semen in concentrated pellet form. *Vth Congress Animal Reproduction, Trento.* 1964. No. 4. P. 410–415.

22. Nava-Trujillo H., Armando Quintero-Moreno A., Geovanny Finol-Parra G., Gabriela Carruyo G., Venuslira Vilchez-Siu V., Carla Osorio-Meléndez C., Jorge Rubio-Guillén J., Valeris-Chacín R. Relationship among damaged chromatin, motility and viability in cryopreserved spermatozoa from Brahman bulls. *Revista Colombiana de Ciencias Pecuarias*. 2011. 24. No 2.

23. Pakėnas P. Veislinių bulių laikymo ir naudojimo Lietuvos technologija. V.: Academia, 1993. P. 18–143.

24. Perez-Garnelo S.S., M. Oter, C. Borque, C. Talavera, M. Delclaux, E. Martinez-Nevado, A.T. Palasz, J. De la Fuente, Post-thaw viability of European bison (*Bison bonasus*) semen frozen with extenders containing egg yolk or lipids of plant origin and examined with a heterologous in vitro fertilization assay, *Journal of Zoo Wildlife Medicine.* 2006. 37 P. 116–125.

25. Pileckas V. Kriokonservavimo technologinių procesų įtaka bulių spermos kokybei: Biomedicinos mokslų habilitacinis darbas. Baisogala, 2002.

26. Sorensen E. Overforing of Gelatineret Sperma iparafinrede cellophanror. *Medlems bldenkske Dyrlaegforen.* 1960. 23. P. 166.

27. Leite T.G., Vale Filho V.R., Arruda R.P., Andrade A.F., Emerick, L.L., Zaffalon F.G. , Martins J.A., and Andrade V.J. Effects of extender and equilibration time on post-thaw motility and membrane integrity of cryopreserved Gyr bull semen evaluated by CASA and flow cytometry. *Animal Reproduction Science*. 2010. 120. P. 31–38.

28. Tabassomi M., Alavi-Shoushtari S. M. Effects of *in vitro* copper sulphate supplementation on the ejaculated sperm characteristics in water buffaloes (*Bubalus bubalis*). *Veterinary Research Forum.* 2013. 4(1). P. 31–36.

29. Van–Wagtendonk–de Leeuw A., Haring R., Kaal–Lansbergen L. et al. Fertility results using bovine semen cryopreserved with extenders based on egg yolk and soy bean extract. *Theriogenology.* 2000. 54. P. 57–67.

30. Watson P. F. The causes of reduced fertility with cryopreserved semen. *Animal Reproduction Science.* 2000. 60–61. P. 481-492.

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