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Influence of medium on viability of bovine embryos produced in vitro after their biopsy

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Influence of mediums following biopsy on the viability of bovine embryos produced in vitro

Summary

The aim of this study was to estimate the viability of biopsied bovine embryos produced *in vitro* in Ham's F10 and IVM media. 52 biopsied embryos (23 morulae, 17 early blastocysts and 12 blastocysts) were cultured for 24 hours in vitro in Ham's F10 and IVM media. 41 (78.85%) embryos were restored: 16 (69.57%) morulae, and 14 (82.35%) early blastocysts and 11 (91.67%) blastocysts ($P \leq 0.05$). Biopsied morulae restored better in the Ham's F10 medium (71.43%) than in the IVM (66.67%) ($P \leq 0.05$). 88.89% of early blastocysts restored in Ham's F10 medium and 75.0% of these embryos restored in IVM medium ($P \leq 0.05$). 100% of blastocysts restored in Ham's F10 medium and 83.33% of them restored in IVM ($P \leq 0.05$). 42.86% of biopsied morulae cultured in Ham's F10 medium and only 11.11% of these embryos cultured in the IVM were of excellent quality. About 2 twice as few embryos were of good quality in Ham's F10 medium (28.57%) than in IVM medium (55.56%) ($P \leq 0.05$). 55,56% of biopsied early blastocysts cultured in Ham's F10 medium and 25,0% of these embryos cultured in IVM medium were of excellent quality ($P \leq 0.05$). 33.33% of good quality early blastocysts were obtained in Ham's F10 medium and 50.0% in IVM ($P \leq 0.05$). 66.67% of biopsied blastocysts cultured in Ham's F10 medium and only 16.67% of these embryos cultured in IVM were of excellent quality. 33.33% good quality blastocysts were obtained in Ham's F10 medium and 66.60% in the IVM ($P \leq 0.05$).

In vitro produced bovine morulae, early blastocysts and blastocysts which restored more when they were cultured in Ham's F10 medium after biopsy. Thus, Ham's F10 medium is more suitable for the viability of biopsied bovine embryos produced in vitro than IVM.

Keywords: bovine, morulae, blastocysts, biopsy

The evaluation of forthcoming offspring's sex was of interest to the farmers for a long time. It is possible to create the population of genetically precious livestock of desirable sex. The sex of offspring's may be estimated from several embryo's blastomeres by the new and most precise method (polimerase chain reaction (PCR)) by which the specific DNA fragments of Y chromosome are determined witch the X chromosome hasn't. Bovine embryos' cells can be used not only for their sex determination but also for investigations of poliploidy, genotyping for κ-casein, growth hormone (GH) and prolactin (PRL) polymorphic alleles, genetic determined diseases (bovine leukocyte adhesion deficiency – BLAD) (7, 9).

Early diagnosis of diseases, which can be inherited genetically, is very significant in human medicine. The biopsy of human embryos and I or II polar body can be made in order to diagnose a1-antitrypsin disease, Down syndrome, Robertsonian translocation, cystic fibrosis, Lesch-Nyhan disease, Tay-Sachs disease, *polyposis coli,* Fragile X syndrome, myotonic dystrophy, sickle cell disease, thalassemia, Fanconi anemia-A, Spinal muscular atrophy-1, retinitis pigmentosa, neurofibromatosis type 1, Huntington disease, Duchenne muscular dystrophy, haemophilia A and B and other diseases (11, 17, 23, 25, 28, 32, 33).

The cells of *in vitro* or *in vivo* produced bovine embryos, which were biopsied (1, 5, 29-31) or separated during splitting of morulae and blastocysts (5, 15), can be used for investigations.

The efficiency of testing and the viability of embryos depend on the number of blastomeres, which were taken by biopsy. Several cells are taken by microaspiration more simply, but the precision of PCR is smaller. When a large number of blastomeres is taken by microsection the viability of embryos decreases (7). Many authors (10, 16, 18, 19, 22) recommend to biopsy 2-15 cells, but Vajta et al. (30) suggests to take 15-20% of blastomeres.

Carbonneau with co-workers (8) proved that *in vitro* produced bovine embryos of 5 days are more sensitive to biopsy if compare to those of 7 days. Vajta (31) maintained that 98% of blastocysts derived *in vitro* survived after biopsy by microsection in their

laboratory and 85% of blastocysts were viable after biopsy and vitrification. Forel et al. (10) estimated that 58% of *in vitro* produced blastocysts survived after biopsy by microaspiration.

The scientists use different media for culture of biopsied bovine embryos: Menezo's B2 (8), TCM-199 with foetal calf serum (24, 30, 31), CR-1aa (2), Dulbecco's phosphate buffered saline (24). There is lack of information in the literature concerning comparison of different media and it is not clear which medium is most suitable for embryos' culture after their biopsy. Therefore in this study we tried to compare the influence of Ham's F10 and IVM media, which were most available to us for the viability of *in vitro* produced bovine embryos after their biopsy.

Material and methods

Production of bovine embryos *in vitro*. Ovaries were obtained from slaughtered cows and heifers. Material was transported and oocytes were collected according to general method (12-14, 20). The Ham's F10 medium, Sigma, Cat. No. N-6635, with 10% foetal bovine serum (FBS) and 10 µg/ml follicle stimulating hormone (FSH) was used for maturation of oocytes (27). Oocytes were matured in medium under mineral oil for 24 h at 38.5°C in 100% air humidity in a special gas atmosphere consisting of 5% O_2, 5% CO_2, 90% N_2. Maturity of oocytes was assessed according to changes in the cumulus layer. Sperm separation was carried out using a „swim-up" method from frozen - thawed bull semen in the Sp/Hepes-TALP medium (26) with 6.0 mg/ml bovine serum albumin (BSA). Oocytes were fertilised for 18 hours in the IVF-TALP medium (3) with 6.0 mg/ml BSA and 20 µg/ml heparin at 38.5°C in 100% air humidity in a special gas atmosphere consisting of 5% O_2, 5% CO_2, 90% N_2. Fertilised oocytes were cultured in the IVM medium (M199 Hepes, Sigma, Cat. No. M-2520, with 10% FBS) in the same conditions as they were matured (27). Development of embryos was estimated at 72 and 144 hours after maturation according to number of blastomeres.

The biopsy of embryos. 64 embryos (28 morulae, 21 early blastocysts and 15 blastocysts) produced *in vitro* were biopsied using „scratched bottom technique" without micromanipulator (4, 6). 61 (95.31%) of them were biopsied successfully: 26 (81.08%) morulae, 20 (95.24%) early blastocysts and 15 (100%) blastocysts. 52 embryos (23 morulae, 17 early blastocysts and 12 blastocysts) were cultured *in vitro* for investigation of their viability after biopsy (tab. 1). Two media were used for culture of embryos: Ham's F10 (Sigma, Cat. No. N-6635, with 10% FBS) and the culture medium IVM. 24 hours cultured embryos were estimated by stereomicroscope and stained with Hoechst 33342 (2 µg/ml) and propidium iodide (10 µg/ml). The number of viable (blue) and dead (pink) blastomeres was determined using fluorescent microscope (MBY 15, Russia). Embryos were classified morphologically for excellent, good and degenerated quality. Embryos that had very small amount (< 10%) of dead blastomeres are classified as excellent quality. Embryos of good quality had 10-20% dead blastomeres. Embryos that had more than 20% dead cells are classified as degenerated (21, 27).

Results were analysed using the Student – Gaset's test.

Results and discussion

The data of fig. 1 show the number of blastomeres that were biopsied from the embryos. 9.77 ± 0.39 blastomeres were taken from morulae during biopsy. 19.1 ± 0.6 cells were biopsied from early blastocysts and 27.93 ± 0.89 cells were taken from blastocysts ($P \leq 0.05$).

Overall 41 (78.85%) embryos restored during 24 hours of culture: 16 (69.57%) morulae, and 14 (82.35%) early blastocysts and 11 (91.67%) blastocysts ($P \leq 0.05$) (tab. 1).

The results of investigations of embryos' restoring show that biopsied morulae restored well in the Ham's F10 medium (71.43%) than in the IVM (66.67%) ($P \leq 0.05$) (fig. 2). 88.89% of early blastocysts restored in the Ham's F10 medium and 75.0% of these embryos restored in the IVM medium ($P \leq 0.05$). 16.67% of blastocysts restored more in the Ham's F10 medium than in the IVM ($P \leq 0.05$).

The data of fig. 3 show the influence of restoring media on the quality of biopsied morulae: 42.86% of biopsied morulae cultured in Ham's F10 medium and

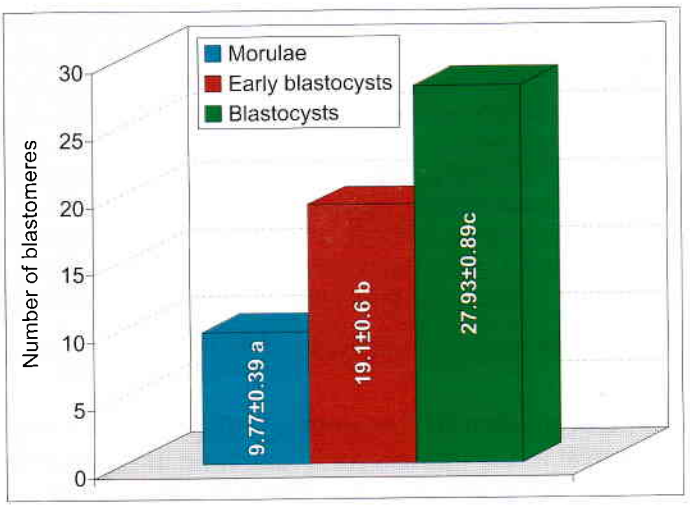

Fig. 1. The number of blastomeres biopsied from bovine embryos produced in vitro ($\bar{x} \pm S_x$)
Explanation: a, b, c – significant differences at $P \leq 0.05$

Tab. 1. The results of the investigation of biopsy and restoring of *in vitro* produced bovine embryos

Procedure	Total	Morulae	Early blastocysts	Blastocysts
Biopsied	64	28	21	15
Biopsied successfully (%)	61 (9.31)	26 (92.86)	20 (95.24)	15 (100)
Collapsed (%)	3 (4.69)	2 (7.14)	1 (4.76)	0
Cultured *in vitro* after biopsy	52	23	17	12
Restored (embryos of excellent and good quality) (%)	41 (78.85)	16 (69.57)[a]	14 (82.35)[b]	11 (91.67)[c]

Explanation: a, b, c – significant differences at $P \leq 0.05$

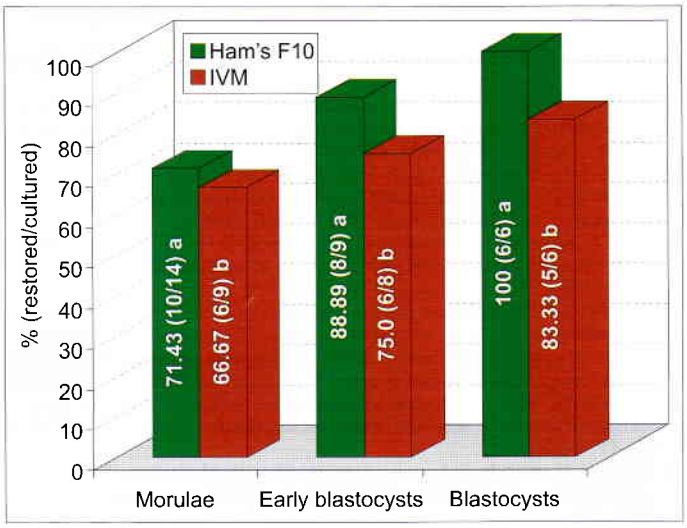

Fig. 2. The influence of media on restoring of *in vitro* produced bovine embryos after biopsy (%)
Explanation: a, b – significant differences at $P \leq 0.05$

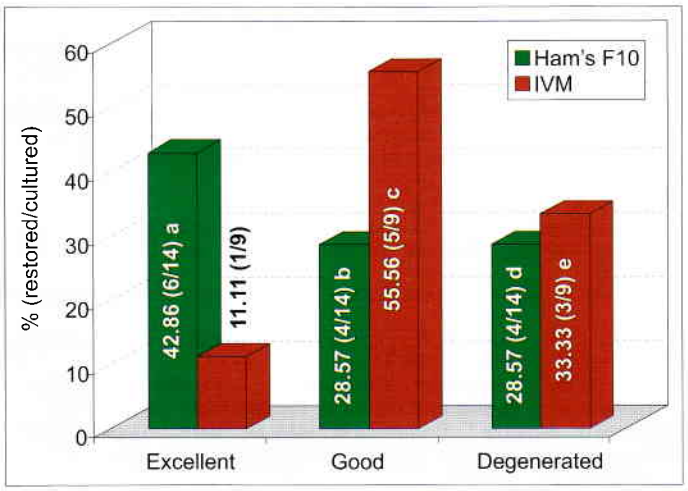

Fig. 3. The influence of media on quality of *in vitro* produced bovine morulae after biopsy (%)
Explanation: a:b, a:d, b:c, c:e – significant differences at $P \leq 0.05$; b:d, d:e – not significant differences at $P \geq 0.05$

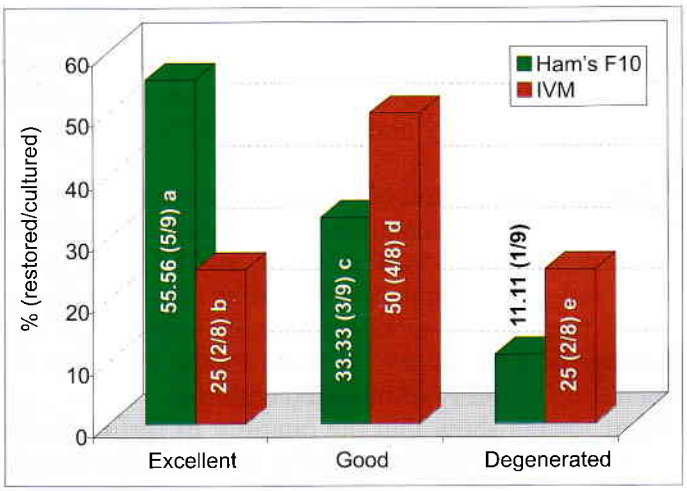

Fig. 4. The influence of media on quality of *in vitro* produced bovine early blastocysts after biopsy (%)
Explanation: a:b, a:c, b:d, c:d, d:e – significant differences at $P \leq 0.05$; b:e – not significant difference at $P \geq 0.05$

only 11.11% of these embryos cultured in the IVM were of excellent quality. About 2 times less of embryos were of good quality in Ham's F10 medium than in IVM medium ($P \leq 0.05$). 28.57% of biopsied morulae cultured in Ham's F10 medium and 33.33% of these embryos cultured in the IVM degenerated ($P \geq 0.05$).

Fig. 4 shows the influence of restoring media on quality of biopsied early blastocysts. About 2 times as much early blastocysts were of excellent quality in Ham's F10 medium than in IVM medium ($P \leq 0.05$). 33.33% of good quality early blastocysts were obtained in the Ham's F10 medium and 50.0% in the IVM ($P \leq 0.05$). Much less of embryos degenerated when they were cultured in Ham's F10 medium (11.11%) than in the IVM (25.0%).

The data of fig. 5 show the influence of restoring media on the quality of biopsied blastocysts: 66.67% of biopsied blastocysts cultured in the Ham's F10 medium and only 16.67% of these embryos cultured in the IVM were of excellent quality. In the Ham's F10 medium blastocysts of good quality were 2 times less than in IVM ($P \leq 0.05$). 16.67% of embryos cultured in IVM medium degenerated.

The offspring's' sex, genotype and genetically determined diseases can be estimated at early embryonic period. The precision of test depends on the number of blastomeres. The biopsy of large number of blastomeres is easier, but the embryos are injured more and therefore develop worse after their transplantation (7, 10, 22). The position of morulae hasn't the account during biopsy. During biopsy of blastocyst the cells are taken from trophectoderm only, the inner cell mass must not be touched. The dead cells can be used for investigation also. But some cells may have mutated DNA; therefore they may be used with viable blastomeres (7). The best results are received when the biopsy is done by microsection and 7-30 blastomeres are ta-

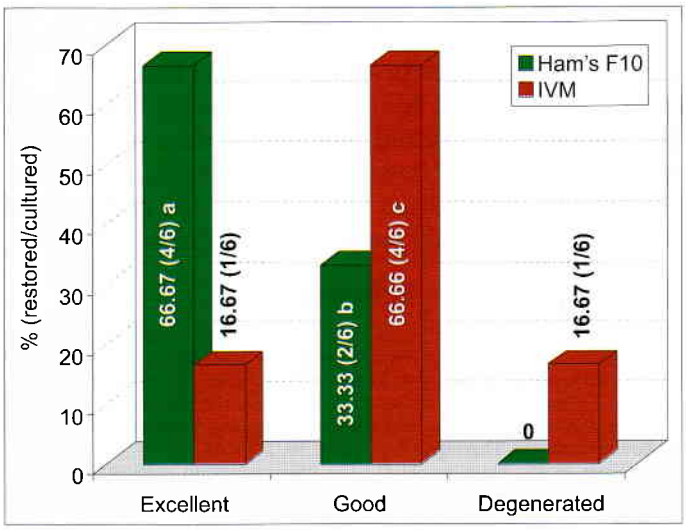

Fig. 5. The influence of media on quality of *in vitro* produced bovine blastocysts after biopsy (%)
Explanation: a:b, b:c – significant differences at $P \leq 0.05$

ken (8, 24). Kirkpatrick and Monson (16) took 2-8 cells. The other authors (10, 18, 19, 22) recommend to biopsy 5-15 cells. Vajta with co-workers (30) suggests to take 15-20% of blastomeres. During our investigations 9.77 ± 0.39 blastomeres were taken from morulae, 19.1 ± 0.6 cells were biopsied from early blastocysts and 27.93 ± 0.89 from blastocysts. Therefore we can suggest that this number of biopsied blastomeres is sufficient for the embryos' genetic analyses.

Nainiene (24) maintained that *in vivo* produced bovine blastocysts are more sensitive to biopsy by microsection than morulae. 100% of morulae and 83.3% of blastocysts were viable when they were cultured after biopsy *in vitro* for 24 hours. After 36 hours of culture 75% of morulae and 66.7% of blastocysts were viable. Agca et al. (1) and Carbonneau et al. (8) demur to these propositions. They estimated that blastocysts of 7 days old were more resistant to biopsy than morulae of 5 days old. According to Vajta's (31) data 98% of blastocysts produced *in vitro* survived after biopsy by microsection. The results of our study coincide with these authors' results (1, 8, 31). *In vitro* produced biopsied bovine blastocysts restored more (91.67%) than early blastocysts (82.35%) and morulae (69.57%).

The Ham's F10 and M199 (as background of IVM) media are produced commercially. Ham's F10 medium has very smaller amount of $CaCl_2$ and KCl and more NaCl and glucose than M199. Tween 80 is only in the M199 medium. It is likely that differences of the quantity of components of these media have the influence on viability of biopsied embryos.

Our results show that the quantity of restored embryos was major when they were cultured after biopsy in Ham's F10 medium (71.43% of morulae, 88.89% of early blastocysts and 100% of blastocysts) than in IVM (66.67%, 75% and 83.33% respectively). The number of excellent and good quality embryos was major in Ham's F10 medium than in IVM. These statistically significant results ($P \leq 0.05$) show that the Ham's F10 medium fits more for the restoring of biopsied bovine embryos produced *in vitro* than IVM.

In vitro produced bovine morulae, early blastocysts and blastocysts restored more when they were cultured in the Ham's F10 medium after biopsy. Therefore the Ham's F10 medium fits more for the restoring of biopsied bovine embryos produced *in vitro* than IVM.

References

1. *Agca Y., Monson R. L., Northey D. L., Peschel D. E., Schaefer D. M., Rutledge J. J.*: Post-thaw survival and pregnancy rates of biopsied, sexed and vitrified bovine IVF embryos. Theriogenology 1995, 43, 153-159.
2. *Agca Y., Monson R. L., Northey D. L., Peschel D. E., Schaefer D. M., Rutledge J. J.*: Normal calves from transfer of biopsied, sexed and vitrified IVP bovine embryos. Theriogenology 1998, 50, 129-145.
3. *Bavister B. D., Yanagimachi D.*: The effect of sperm extracts and energy sources on the motility and acrosome reaction of hamster spermatozoa in vitro. Biol. Reprod. 1977, 16, 228-237.
4. *Bredbacka P., Kankaanpaa A., Peippo J.*: PCR-sexing of bovine embryos: a simplified protocol. Theriogenology 1995, 44, 167-176.
5. *Bredbacka P., Velmal R., Peippo J., Bredbacka K.*: Survival of biopsied and sexed bovine demi-embryos. Theriogenology 1994, 41, 1023-1031.
6. *Bredbacka P.*: Biopsy of morulae and blastocysts. Reprod. Dom. Anim. 1991, 26, 82-84.
7. *Bredbacka P.*: Recent developments in embryo sexing and its field application. Repr. Nutr. Dev. 1998, 38, 605-613.
8. *Carbonneau G., Morin N., Durocher J., Bousquet D.*: Viability of bovine IVF embryos biopsied with microsection or microaspiration technique for sexing. Theriogenology 1997, 47, 266-271.
9. *Chrenek P., Boulanger L., Heyman Y., Uhrin P., Laurincik J., Bulla J., Renard J. P.*: Sexing and multiple genotype analysis from a single cell of bovine embryo. Theriogenology 2001, 55, 1071-1081.
10. *Forel F., Lopes R. F. F., Rodrigues J. L.*: Sexing of in vitro produced bovine embryos using pit-stop PCR after microaspiration (abstract). Theriogenology 2002, 57, 747.
11. *Harper J.*: Preimplantation genetic diagnosis. In-vitro News 1998, 9, 2-3.
12. *Hashimoto S., Saeki K., Nāgao Y., Minami N., Yamada M., Utsumi K.*: Effects of cumulus cell density during in vitro maturation on the developmental competence of bovine oocytes. Theriogenology 1998, 49, 1454-1463.
13. *Hawk H. W., Wall R. J.*: Improved yields of bovine blastocysts from in vitro-produced oocytes. I. Selection of oocytes and zygotes. Theriogenology 1993, 40, 1571-1583.
14. *Hawk H. W., Wall R. J.*: Improved yields of bovine blastocysts from in vitro-produced oocytes. II. Media and co-culture cells. Theriogenology 1993, 41, 1585-1594.
15. *Kippax I. S., Christie W. B., Rowan T. G.*: Effects of method of splitting, stage of development and presence or absence of zona pellucida on foetal survival in commercial bovine embryo transfer of bisected embryos. Theriogenology 1991, 35, 25-35.
16. *Kirkpatrick B. W., Monson R. L.*: Sensitive sex determination assay applicable to bovine embryos derived from IVM and IVF. J. Reprod. Fertil. 1993, 98, 335-340.
17. *Kuliev A., Rechitsky S., Verlinsky O., Strom C., Verlinsky Y.*: Preembryonic diagnosis for sickle cell disease. Mol. Cell Endocrinol. 2001, 183, Suppl. 1, 19-22.
18. *Lacaze S., Lesclaux J., Coupet H.*: The sexing of bovine embryos in South-West of France: I. Efficiency, accuracy of size and pregnancy rates after three years of activity (abstract). Proc. 12th Annual Meeting of the AETE. Lyon 1996, p. 156.
19. *Lacaze S., Lesclaux J., Coupet H.*: The sexing of bovine embryos in South-West of France: II. Influence of size of biopsy on sex efficiency and pregnancy rates (abstract). Proc. 12th Annual Meeting of the AETE. Lyon 1996, p. 158.
20. *Larsson B., Jaakma U.*: IVM/IVF/IVC manual for bovine oocytes. Uppsala 1994.
21. *Linder G. M., Wright R. W.*: Bovine embryo morphology and evaluation. Theriogenology 1983, 20, 407-414.
22. *Lopes R. F. F., Termignoni C., Rodrigues J. L.*: Sex determination of bovine embryos using Pit-stop PCR (abstract). Theriogenology 2000, 53, 482.
23. *Munne S., Escudero T., Sandalinas M., Sable D., Cohen J.*: Gamete segregation in female carriers of Robertsonian translocations. Cytogenet. Cell Genet. 2000, 90, 303-308.
24. *Nainiene R.*: The effect of biopsy on viability and sex determination of bovine embryos. PhD thesis. Lithuanian Institute of Animal Science, Baisogala 1999.
25. *Ouhibi N., Olson S., Patton P., Wolf D.*: Preimplantation Genetic Diagnosis. Current Women's Health Rep. 2001, 1, 138-142.
26. *Parrish J. J., Susko-Parrish J., Leibfried-Rutledge M. L., Critser E. S., Eyestone W. H., First N. L.*: Bovine in vitro fertilization with frozen-thawed semen. Theriogenology 1986, 25, 591-600.
27. *Rho G. J., Johnson W. H., Betteridge K. J.*: Cellular composition and viability of demi- and quarter-embryos made from bisected bovine morulae and blastocysts produced in vitro. Theriogenology 1998, 50, 885-895.
28. *Strom C. M., Ginsberg N., Rechitsky S., Cieslak J., Ivakhenko V., Wolf G., Lifchez A., Moise J., Valle J., Kaplan B., White M., Barton J., Kuliev A., Verlinsky Y.*: Three births after preimplantation genetic diagnosis for cystic fibrosis with sequential first and second polar body analysis. Am. J. Obstet. Gynecol. 1998, 178, 1298-1306.
29. *Tominaga K.*: Cryopreservation and sexing of in vivo- and in vitro-produced bovine embryos for their practical use. J. Reprod. Dev. 2004, 50, 29-38.
30. *Vajta G., Holm P., Greve T., Callesen H.*: Comparison of two manipulation methods to produce in vitro fertilized, biopsied and vitrified bovine embryos. Theriogenology 1997, 47, 501-509.
31. *Vajta G.*: Bovine in vitro production, biopsy and cryopreservation. PhD thesis. Danish Institute of Agricultural Sciences, Department of Animal Breeding and Genetics, Copenhagen 1997.
32. *Verlinsky Y., Kuliev A.*: Preimplantation polar body diagnosis. Biochem. Mol. Med. 1996, 58, 13-17.
33. *Verlinsky Y., Rechitsky S., Cieslak J., Ivakhnenko V., Wolf G., Lifchez A., Kaplan B., Moise J., Walle J., White M., Ginsberg N., Strom C., Kuliev A.*: Preimplantation diagnosis of single gene disorders by two-step oocyte genetic analysis using first and second polar body. Biochem. Mol. Med. 1997, 62, 182-187.

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