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# Comparative evaluation of the cattle demi-embryos produced in vitro and in vivo

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Summary

**The aim of this study was to split the morulas and blastocysts produced in vitro, to cultivate them in vitro, to evaluate their viability and to compare them with those produced in vivo.**

**Ovaries were obtained from slaughtered cows and oocytes were aspirated according to generally accepted methods. Maturation was conducted at 38.5°C with 100% humidity for 24 hours in 500 ml of the maturation medium under mineral oil, in the pores of plates. The spermatozoids were selected by the schwim-up method. The in vivo embryos were obtained according to the generally accepted methods of transplantation. Embryos were washed by the nonsurgical method with a Kassou catheter on the 6-7th days after fertilization. The embryos were split in a drop of Dulbecco phosphate buffer solution (PBS) according to the „scrathed bottom" technique without a micromanipulator. They were split by a microknife made from a shaving blade. The differentiated embryos were split taking into account the conditional axis of symmetry crossing the embryoblast and trophoblast. The cultivated halves of the embryos were stained at room temperature for 30 minutes with Hoechst 33342 (2 μg/ml) dye and propidium iodide. The evaluation was performed by a MBI –15 microscope in a fluorescent regime.**

**In vitro produced demi-embryos were weaker than those produced in vivo and had a lower level of reparation. The output of valuable demi-embryos decreased because reparation failed in 36.0% of the in vitro obtained demi-embryos. During the cultivation of the in vivo produced demi-embryos degeneration was observed in 7.5% of cases. Splitting caused damage to 29.44% of the blastomeres from the morulas and 23.98% of the blastomeres from the blastocysts. Ham's F10 medium is more suitable for the reparation of early embryos than IVM medium.**

**Keywords: bovine, splitting, embryos**

Splitting of embryos is generally used in order to obtain identic twins from the same female and produce possibly higher number of calves (12, 16, 17, 19). The quality of the calves is greatly influenced by the stage of embryo splitted and the technique used as well as the technique of transplantation (3, 10). During the microsurgical operation the structure of embryo is destroyed and contacts among the cells are disturbed. This process is especially clear observed while splitting blastocysts. These embryos are able to form a normal embryo while cultivating *in vitro* (11). Undamaged blastomeres are able to phagocyte degenerated cells and their fragments and the structure of embryo halves is regained in 30 minutes (2). It is stated that the embryos of excellent quality, cultivated for 48 hours *in vitro* make 50% of the cells in intact embryo (13). Embryos produced *in vitro* can be splitted as successfully as the ones produced *in vivo*.

The embryos separated during the process of splitting can be used for the PRG reaction. It is necessary to stress that their quality is lower than of the embryos produced *in vivo*. After surgical intervention they require special conditions of cultivation. The halves of *in vitro* produced embryos are cultivated in TMC 199 medium conditioned with the epithelium cells from the cows' oviduct., PBS with fetal serum of calves or inactivated blood serum from rutting cows (15).

The aim of this study was to split the morulas and blastocysts produced *in vitro*, to cultivate them *in vitro*, to evaluate their viability and to compare them with those produced *in vivo*.

## Material and methods

Ovaries for the fertilization were obtained from the slaughtered cows. The ovaries were transported and the oocytes were aspirated according to generally accepted methods (6). Ovaries were washed three times in 0.9% NaCl solution supplemented with 0.1 g/l streptomycin, 100 000 VV/l penicillin at 38°C temperature. The fluid of oocytes was kept for 10 minutes in the manipulation medium (M199 Hepes). A stereomicroscope was used for the selection of the oocytes. The cells selected later on were washed three

times in the manipulation medium. Only the oocytes with homogenic, evenly spread ooplasm, an incomposite permanent involucre and multi-layer, adherent cumulus were used in the experiments. The maturation was carried out at 38.5°C temperature, 100% humidity for 24 hours in 500 ml of the maturation medium under the mineral oil, in the pores of plates (Nunc 1767400). The maturation was evaluated according to the changes in the cumulus layer. (It must have been tripled and unseparated during washing). The bulls sperm frosen in special straws was used for the fertilization. The spermatozoides were selected by the schwim--up method. The straws were thawed for 15 seconds at 34°C temperature water. The procedure was carried out in the 2 ml volume cryotest-tubes (Roth E 309. 1). 200 µl of sperm were injected under the capacitation medium (1 ml). The test – tubes canted in 45° angle were incubated at 38.5° temperature for 60 minutes. Then the upper layer of the medium about 350 µl) was sucked and it was centrifuged twice for 10 min. with 1000 rot/min. The centrifugate was used for the fertilization *in vitro*. The maturated oocytes were washed twice with the fertilization medium. 1-1,5·$10^6$ of spermatozoids were added to the 500 µl of this medium with oocytes and mineral oil was poured. The cultivation was carried out for 14 hours at 38.5°C temperature, 100% humidity. After the procedure of fertilization the oocytes were washed with the cultivation medium and cultivated at 38.5°C temperature in 500 µl of the same medium, under the mineral oil layer. 200 µl of the medium were changed after 72 and 144 hours. The development of embryos was evaluated according to the number of blastomeres produced. All the biotechnological procedures were carried out under the special gas medium, consisting of 5% $O_2$, 5% $CO_2$ and 90% $N_2$.

The *in vivo* embryos were obtained according to the generally accepted methods of transplantation. The superovulation was provoked with the help of hypophysis extract from pigs, which served as the gonadotropic hormone under the comercial title „Follitropin". The preparation was injected twice daily into the muscle of the cows every twelve hours on the $10^{th}$ day of the sexual cycle, if the corpus luteum was evidently observed. The 4 day donor treatment scheme was used. The total dose of preparation was 1200 VV. During the first day 300 vv were injected, during the second – 300 vv, during the third – 450 vv and during the fourth – 150 vv. The timing of ovulation and rutting was reached on the third day of injection using the analogous of prostoglandine $F_2\alpha$ – cloprostenol – 0.25 mg (7). The cows were inseminated twice with the interval of 12 hours using cryopreserved sperm 56 hours after the injection of prostaglandine. One dose contained 20 millions of spermatozoids. The embryos were washed by the nonsurgical method with a Kassou catheter on the 6-7-th day after fertilization. For the washing-up of the embryos from one donor was used 1000 ml PBS with 1% foetal serum of calves. The medium was kept in the room temperature for 15 min., the embryos were collected by the plastic caplillary of the Cassou firm for the embryos. Only high quality morulas and blastocysts produced *in vivo* and *in vitro* were used in the experiments. (High quality embryos are considered to be the ones, the stage of development of which correspond to the time of cultivation, with no signs of damaged and degenerated blastomeres). The embryos were splitted in the drop of Dulbecco phosphate buffer solution (PBS) according to the technique of „scrathed bottom" (3, 14) without a micromanipulator. The bottom of the Petri plate was scratched with an injective needle. The embryo was placed at the bottom of the plate in the drop of room temperature and fixed on the scratches. It was splitted by the micro knife made of shaving blade. The embryo was oversquashed into possibly equal parts. The differentiated embryos were splitted taking into account the conditional axis of symmetry, crossing embryoblast and trophoblast. The manipulation was considered to be successful if both halves of the embryos were practically equal, compact, only few blastomeres were destroyed. After the procedure PBS with 10% of foetal serum of calves was added to the drop of medium in order to cause the segregation of the embryo halves. On the whole, 74 *in vitro* and 36 *in vivo* produced embryos were splitted. The demi-embryos were transferred into the reparation medium and cultivated for 24 hours under the mineral oil layer. Ham's F10 and IVM media without hormones were used for the reparation. The cultivated halves of the embryos were stained at room temperature for 30 minutes with Hoechst 33342 (2 µg/ml) dye and propidium iodide. The evaluation was performed by a microscope MBI–15 in fluorescent regime. The alive (shine bluishy) and damaged during splitting (shine rosy) blastomeres were counted. The halves with up to 10% of degenerated cells were considered to be excellent (13).

The results of the investigations were evaluated statistically using EXCEL 2000 programme according to the criteria of Stjudent-Gaset.

## Results and discussion

61 (82.43%) *in vitro* and 30 (83.23%) *in vivo* produced embryos were splitted successfully. From the embryos produced *in vitro* we succeeded to split 30 (49.18%) morulas and 31 (50.81%) blastocysts. 122 halves with visually good structure were obtained. 100 halves of embryos (50 morulas, 50 blastocysts) were cultivated *in vitro*. From the embryos produced *in vivo* 14 (77.78%) demi-morulas and 16 (88.89%) demi-blastocysts were produced. We obtained 60 visually valuable halves. 40 halves of these embryos (20 morulas and 20 blastocysts) were cultivated *in vitro*. During 24 hours of cultivation the recovery was observed in 64 (64.0%) *in vitro* produced and 37 (92.5%) *in vivo* produced demi-embryos. In case of *in vitro* produced embryos the recovery was observed in 31 (48.43%) halves of morulas and in 24 (37.5%) halves of blastocysts. In case of *in vivo* produced embryos the reparation occurred in 19 (51.3%) halves of morulas and in 18 (48.64%) halves of blastocysts. (tab. 1).

As the results in table 1 evidently demonstrate, about one fifth of embryos are destroyed during splitting. The destruction was in 0.8% higher in case of *in vitro* produced embryos, but this difference was no statistically reliable ($p > 0.05$). After 24 hours of cultivation the number of repaired demi-embryos produced of *in vivo*

**Tab. 1. Results of microsurgery of *in vitro* or *in vivo* produced embryos**

| Manipulation | *In vitro* produced embryos | | | *In vivo* produced embryos | | |
|---|---|---|---|---|---|---|
| | Total | Morulas | Blastocysts | Total | Morulas | Blastocysts |
| Splitted | 74 | 37 | 37 | 36 | 18 | 18 |
| Destroyed during splitting (%) | 13 (17.57) | 7 (18.92) | 6 (35.58) | 6 (16.77) | 4 (22.22) | 2 (11.11) |
| Cultivated | 100 | 50 | 50 | 40 | 20 | 20 |
| Recovered demi-embryos (%) | 64 (64.00) a | 31 (62.00) c | 33 (66.00) d | 37 (92.50) b | 19 (95.00) | 18 (90.00) |

Explanations: a:b, c:d, $p \leq 0.05$

**Tab. 2. Influence of the media on development of demi-embryos (%)**

| State embryos | Halves obtained from *in vitro* produced embryos | | Halves obtained from *in vivo* produced embryos |
|---|---|---|---|
| | Ham's F10 medium | IVM medium | Ham's F10 medium |
| Morulas | 70.97 | 47.37 | 95.0 |
| Blastocysts | 84.65 | 51.35 | 90.0 |

**Tab. 3. Number of viable blastomeres obtained from the *in vitro* produced demi-embryos ($\bar{x} \pm SD$)**

| Medium | Demi-embryos obtained from morulas | | Demi-embryos obtained from blastocysts | |
|---|---|---|---|---|
| | After splitting | After reparation | After splitting | After reparation |
| Ham's F10 | 13.30 ± 0.52 | 19.17 ± 0.61 a | 32.00 ± 0.75 | 42.75 ± 2.18 c |
| IVM | 13.20 ± 0.45 | 17.50 ± 0.75 b | 31.20 ± 1.32 | 40.56 ± 1.32 d |
| Comparing to all blastomeres (%) | 70.56 ± 4.28 | 92.95 ± 0.55 | 76.02 ± 4.57 | 91.51 ± 0.55 |

Explanations: a:b, c:d, $p \leq 0,05$

embryos was in 28.5% higher ($p < 0.05$). The recovery of the demi-embryos obtained from *in vitro* produced blastocysts was in 4% better, than from morulas ($p < 0.05$). The recovery of the demi-embryos obtained from morulas was in 5% better than those produced from blastocysts ($p > 0.05$) (tab. 2).

It is evident from the table 2 that Ham's F10 medium caused the recovery of more demi-embryos. From *in vitro* obtained halves of morulas in Ham's F10 medium recovery was observed in 23.6% more cases than in IVM medium ($p < 0.05$), and in case of the halves of blastocysts – in 33.3% more ($p < 0.05$). From *in vivo* obtained halves of morulas in Ham's F10 recovered in 24.03% more than from obtained *in vitro* ($p < 0.05$). The recovery of the *in vitro* obtained blastocysts in this medium was in 5.35% lower than of those obtained *in vivo* ($p > 0.05$). The level of reparation of the blastocysts produced *in vivo* was in 5.0% ($p > 0.05$) lower if to compare to the morulas. (tab. 3).

As it is demonstrated in the table 3, splitting of morulas cause destruction of 29.44% blastomeres and splitting of blastocysts – 23.98% blastomeres. Cultivation of demi-embryos increase the number of viable cells. Demi-embryos obtained from morulas after 24 hours of cultivation had in 30.62% more blastomeres if to compare to the splitted ones. Blastocysts had in 25.14% more ($p > 0.05$) blastomeres after the reparation. As in Ham's F10, so in IVM media the number of alive blastomeres from cultivated morulas was as many as 14 times, the number of alive halves of blastocysts increased on average in 1.33 times. The number of blastomeres from morulas after the reparation in Ham's F10 medium was in 9.54% higher than in case of the reparation in IVM medium ($p < 0.05$). The number of blastomeres from the blastocysts after the reparation in F10 medium was in 5.39% higher than in IVM medium ($p < 0.05$).

Quite effective splitting of the early embryos can be reached using a microknife without the help of a micromanipulator. *In vitro* produced demi-embryos are weaker than those produced *in vivo* (18). The disturbed development of the embryos is described as the large offspring syndrome, in spite of the fact that the reasons of this phenomenon are not known exactly (8, 9) This fact can explain the lower output of the demi-embryos produced *in vitro* and lower level of the reparation. The output of valuable demi-embryos decreased because the reparation failed in 36,0% of the *in vitro* obtained demi-embryos. During the cultivation of the *in vivo* produced demi-embryos the degeneration was observed in 7.5% of cases. The cultivation in the same condition (Ham's F10) lead to the conclusion that the reparation of the *in vitro* produced demi-embryos from morulas was in 24% lower than of the *in vivo* produced. This difference between the *in vivo* and *in vitro* produced blastocysts was 5.35%). The microknife manipulation cause the damage of about 25-30% blastocysts and 20% of morulas. (4). The data of our experiments evidently demonstrated that splitting caused the damage of 29.44% blastomeres from the morulas and 23.98% blastomeres from the blastocysts. The demi-embryo from the morula consisted of 20.17 ± 0.87, and from the blastocyst – 32.0 ± 0.75 blastomeres. The relative number of the alive blastomeres was in 5.46% higher in the demi-embryos from the blastocysts. These data confirm the data of Y. Agca and others (1995) and G. Carbonneau and others (1997) according to which the 7 day blastocysts demonstrate higher resistance to the microsurgical intervention than 5 day morulas. In experiment during cultivation of the demi-embryos the

number of the blastomeres increased if to compare to the splitted embryos. It demonstrates that embryos regain their structure. The output of the embryos depend not only on the stage of the demi-embryos development but on the conditions of the reparation as well. The embryos in Ham's F10 medium consisted of in 7.46% more blastomeres than in IVM medium. It demonstrates once more that Ham's F10 medium is more suitable for the reparation of early embryos.

## References


1. *Agca Y., Monson R. L., Northey D. L., Peschel D. E., Schaefer D. M., Rutledge J. J.*: Posthaw survival and pregnancy rates of biopsied, sexed and vitrified bovine IVF embryos.Theriogenology 1995, 43, 153-159.
2. *Albihn A., Rodriguez-Martinez H., Gustafsson H.*: Morphology of day 7 bovine demiembryos during in vitro reorganization. Acta Anat. 1990, 138, 42-49.
3. *Bredbacka P.*: Recent developments in embryo sexing and its field application. Repr. Nutr. Dev. 1998, 38, 605-6134.
4. *Bredbacka P., Kankaanpaa A., Peipo J.*: PCR-sexing of bovine embryos: simplified protocol. Theriogenology 1995, 41, 1023-10315.
5. *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.
6. *Hashimoto S., Saeki K., Nagao 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.
7. *Jaśkowski J. M., Zbylut J.*: Efficacy of superovulation in cows with respect to time of prostaglandin application. Medycyna Wet. 2000, 56, 541-612.
8. *Yong L. E., Sinclair K. D., Wilmut I.*: Large offspring syndrome in cattle and sheep. Rev. Repr. 1998, 3, 155-163.
9. *Kątska L., Kania G., Ryńska B., Gajda B., Smorąg Z.*: Offspring after transferring goat embryos originating from in vitro matured and fertilised oocytes. Medycyna Wet. 2002, 58, 462-464.
10. *Lopes R. F. F., Forell F., Oliveira A. T. D., Rodrigues J. L.*: Splitting and biopsy for bovine embryo sexing under field conditions. Theriogenology 2001, 56, 1383-1392.
11. *Nimbart M., Sripongpun S., Cedden F., Mechekour F., Le Guienne B., Thibier M.*: Histological study of bovine intact and demi-embryos. Theriogenology 1988, 29, 283-289.
12. *Ozil J. P., Heyman Y., Renard J. P.*: Production of monozygotic twins by micromanipulation and cervical transfer in the cow. Vet. Rec. 1982, 110, 126-127.
13. *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.
14. *Rho G. J., Kawarsky S., Johnson W. H., Kochhar K., Betteridge K. J.*: Sperm and oocyte treatments to improve the formation of male and female pronuclei and subsequent development following intracytoplasmic sperm injection into bovine oocytes. Biol. Reprod. 1998, 50, 885-895.
15. *Riedl J., Zakhartchenko V., Wolf E.*: Effect of embryo developmental stage and quality on the efficiency of in vitro produced bovine embryo splitting. Theriogenology 1996, 45, 221-223.
16. *Skrzyszowska M., Smorąg Z., Kątska L.*: Demi-embryo production from hatching of zona-drilled bovine and rabbit blastocysts. Theriogenology 1997, 48, 551-557.
17. *Williams T. J., Elsaden R. F., Seidel G. E.*: Effect of embryo age and stage on pregnancy rates from demiembryos. Theriogeneology 1984, 26, 276-301.
18. *Wood C.*: Embryo splitting: a role in infertily? Reprod. Fertil. Dev. 2001, 13, 91-93.
19. *Žilaitis V.*: Production and development of monozygotic twins in the cow. Doctoral thesis, Moscow 1989, p. 56-80.


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