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# Effect of Two-step Vitrification on Developmental Competence of *in vitro* and *in vivo* Produced Bovine Embryos

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**Abstract**

The aim of this study was to establish the effect of two-step vitrification on survival rate of bovine embryos produced *in vitro* (method A) and *in vivo* (method B) from Holstein-Friesian cattle. The embryos suitable for vitrification were frozen by a two-step technique, using increasing concentrations of dimethyl sulphoxide (DMSO) and ethylene glycol (EG). After thawing, the quality grade and developmental stage of embryos was assessed. *In vitro* developmental competence of embryos of different quality grade obtained by method B (n = 82) was significantly higher ($p < 0.001$) compared to method A (n = 98). The best results were detected when we vitrified the embryos of the grade 1 quality; namely, the hatched blastocyst stage was reached by 6.9% (2/29) of embryos retrieved by method A and by 36.7% (11/30) of embryos retrieved by method B ($p < 0.01$). In the case of developmental competence of embryos at different developmental stages we reached significantly better results ($p < 0.001$) when we vitrified the embryos produced by method B (n = 84) in comparison with method A (n = 67). We noted a higher hatching rate at the stage of expanded blastocyst; namely, the hatched blastocyst stage was reached by 7.4% (2/27) of embryos produced by method A and by 30.8% (8/26) of embryos produced by method B ($p < 0.05$). In general, the hatched blastocyst stage was reached by 15.1% (50/331) of all thawed embryos retrieved by method A and B.

In conclusion, when we applied two-step vitrification on the grade 1 quality embryos at the stage of expanded blastocyst produced *in vitro* or at the stage of morula produced *in vivo* we achieved the highest hatching rates.

*Cryoconservation, freezing, thawing, cattle embryo, survival rate*

In recent years, there has been a growing need for development and improvement of assisted reproduction methods, in both human and veterinary medicine. In terms of manipulation, trading and conservation of genetic material, the area of cryopreservation has been mainly investigated (Ledda et al. 2005). First theories of cell cryopreservation were established by Mazur (1965) and these were later applied to embryos of mice (Whittingham et al. 1972) and other species (Willadsen et al. 1977; Willadsen et al. 1978; Fahning and Garcia 1992; Dobrinsky et al. 2002; Cuello et al. 2004). In humans, the first pregnancy derived from a frozen embryo occurred in 1983 (Trounson and Mohr 1983). In 1986, Chen (1986) reported the successful cryopreservation of human oocytes. For freezing cryoprotectants that protect against chilling injury are used. They minimize the rise of intracellular ice crystals by removing cytoplasmic water based on the osmotic effect or they interfere with nucleation and ice crystals growing inside or outside the cells. In general, combinations of two or more cryoprotectants are used because of total osmolarity and toxic effect reduction (Ali and Shelton 1993). Cryopreservation methods can be divided into two basic groups: (1) slow (conventional) freezing and (2) rapid freezing (vitrification). Despite the fact that conventional freezing is the most widely used method of cryopreservation of *in vitro* and *in vivo* derived embryos, vitrification method has also been tested in different species with good results (Kuwayama et al. 1992; Vajta 1997; 1998; Berthelot et al. 2000; Lopatarova et al. 2002; Martinez et al. 2006).

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In general, vitrification differs from conventional freezing in a few ways. These are mainly the type or cryoprotectant combination, concentration, volume and exposure time. Vitrified samples are cryopreserved by a faster decrease of temperature (2 000-20 000 °C/min; Martino et al. 1996; Arav and Zeron 1997) in contrast to slow freezing (2 500 °C/min; Rall 1987). It means that only reduced numbers of ice crystals can grow up and cell injuries are minimized. To make the cryopreservation more efficient, it is necessary to understand all the factors that influence this method, e.g. the size of a cryopreserved sample, stage of development, composition of the medium and finally, the method of freezing and thawing. Although a few methods of cryopreservation have been proposed in the last decades, no standardized procedure provided considerable success. The final *in vitro* survival of cryopreserved embryos is insufficient at present.

The aim of this study was the investigation of the vitrification method effect on the survival rate and developmental competence of bovine embryos under *in vitro* conditions. The study was based on the hypothesis of survival rate and cryopreservation efficiency enhancement by using a two-step vitrification method.

## Materials and Methods

The embryos for this experiment were retrieved from Holstein-Friesian cows by two methods.

### *In vitro* embryo production (method A)

In method A the oocytes were obtained from the ovaries of slaughtered cows. In a local slaughterhouse the ovaries were placed into saline (0.9% sodium chloride) at 22-25 °C, transported to the laboratory and processed within 2 h of collection. Firstly, they were washed in alcohol to ensure effective disinfection and then washed twice in saline. Oocytes from the follicles 3-8 mm in diameter were aspirated manually using a 20-gauge needle attached to a 10 ml syringe. All the follicular fluid was allowed to settle at room temperature and the sediment was checked by meandric technique under a stereomicroscope. Only the high-quality oocytes with compact COCs (cumulus oocytes complexes) around *zona pellucida*, i.e. oocytes with more than three layers of compact cumulus cells, were selected and washed two-times in inhibition medium with FBS (foetal bovine serum) and once in maturation medium (TCM-199, tissue culture medium). For maturation, COCs were cultured in four-well dishes (30-50 oocytes per well, NUNC) with 400 µl of maturation medium for 22-24 h at 39 °C, 5% $CO_2$, 7% $O_2$ and 90-95% Rv (Binder). For *in vitro* fertilization (day 0), only verified and tested insemination doses were used. Capacitation and selection of motile spermatozoa was done by the swim-up technique (Parrish et al. 1986): 100 µl of a thawed bovine insemination dose was placed under 1 ml of capacitation medium (sodium pyruvate, bovine serum albumin, gentamicine) and placed for 45 min into the incubator. Afterwards, the supernatant was centrifuged for 15 min at 200 *g* and the volume of sperm suspension to be added to the oocytes to the final concentration 1 mil spermatozoa/ml was calculated from the sediment. Just before addition of spermatozoa, the oocytes were washed twice in fertilization medium (bovine serum albumin, pyruvate) with heparin. The inseminated oocytes were finally kept on a four-well plate and overlain with mineral oil (Sigma-Aldrich). The incubation lasted for 20-22 h. For removing of the cells around zona pellucida, we used a mechanical method of vortexing for 90 s in vortex medium (VIGRO). Afterwards, the embryos were washed twice with the culture medium (CR1), placed into a four-well plate, overlaid with mineral oil and stored at 39 °C, 5% $CO_2$, 7% $O_2$, 90-95% Rv for 7-8 days. After that the quality grades and developmental stages were classified. Embryos suitable for cryopreservation were frozen by a two-step method.

### *In vivo* embryo production (method B)

In method B the embryos were retrieved from superovulated cows. Selected donors were stimulated according to the protocol by Holy et al. (1990). Briefly, Holstein-Friesian cows were superovulated between days 8-12 of the oestrus cycle with eight decreasing doses of 450 I.U. of FSHp (follicle stimulating hormone) and 450 I.U. of LHp (luteinizing hormone) *pro toto* (Pluset®, Calier, Spain) administered at 12-h intervals. Oestrus was induced by double prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) treatment (cloprostenol, Oestrophan®, Bioveta, Czech Republic) together with the fifth and sixth FSH injection. Three artificial inseminations were performed at 48, 60 and 72 h after the first application of $PGF_{2\alpha}$. At day 7-8 the embryos were transcervically flushed from the uterine horns by Dullbecco's phosphate buffer saline (PBS). The flushing fluid was allowed to settle at room temperature and the sediment was checked by meandric technique under a stereomicroscope. After that the quality grades and developmental stages were classified. Embryos suitable for cryopreservation were frozen by a two-step method.

### Vitrification procedure

We used a combination of two cryoprotectants, dimethyl sulphoxide (DMSO) and ethylene glycol (EG) in MOPS solution (modified rich buffer, potassium phosphate), for two-step cryoconservation. The vitrification procedure was performed at room temperature (20-25 °C). Firstly, a 7-min equilibration in 20 µl drop of equilibration solution (ES; MOPS, 7.5% DMSO and 7.5% EG) was performed, and then embryos, with a minimal

volume of ES, were transferred into 20 µl drop of vitrification solution 1 (VS1; MOPS, 15% DMSO and 15% EG) for 7 s. After that the embryos were transferred with a minimal volume of VS1 into the second 20 µl drop of vitrification solution 2 (VS2; MOPS, 15% DMSO and 15% EG) for the same time. Finally, the embryos were transported with a minimal volume of VS2 into 20 µl drop of vitrification solution 3 (VS3; MOPS, 15% DMSO and 15% EG) for 7 s. Within 1 min, embryos in a minimal volume of VS2 (< 1 µl) were transferred to the bottom of the vitrification straw and plunged into liquid nitrogen (-196 °C). No more than two embryos in one procedure were transferred and every procedure was done in fresh drops of ES and VS.

Warming procedure

The straws were thawed after at least one-week storage in liquid nitrogen. The embryos were immediately transferred into 40 µl of warming solution (WS1) with 1 M sucrose for 1 min and then into 20 µl of warming solution (WS2) with 0.5 M sucrose two times for 2 min. Finally, they were placed into 20 µl of MOPS three times for 3 min. The temperature of the media was 37 °C in each of the steps. We used a heated microscope stage. No more than two embryos in one procedure were transferred and every procedure was done in fresh drops of WS. The morphological quality and development stage were evaluated and embryos were transferred into the culture solution CR1 under the same culture conditions as described above. In 48 h cleavage and blastocyst formation were classified based on quality grade and developmental stage.

Embryo assessment

The embryos were classified before cryopreservation and after thawing on the base of the morphological quality. Embryo grading scheme: quality grade 1 (> 85% of morphologically intact intracelullar embryonic mass, ICM; compacted embryos with uniform blastomers and smooth zona pellucida), quality grade 2 (> 50% of morphologically intact ICM; small deviations, e.g. a few excluded blastomeres), quality grade 3 (> 25% of morphologically intact ICM; irregularities in shape, size or colour of ICM). The developmental stages were divided into four grades: morula (32-64 blastomeres), early blastocyst (blastocoel < 50%), blastocyst (blastocoel > 50%) and expanded blastocyst (discernible ICM and trophoblast, thinner *zona pellucida*).

Statistical analysis

The obtained data were analyzed by the $\chi^2$ test (Chi-square test, 2 × 2 contingency tables) and Fisher exact probability test. The results of our study are summarised in Tables 1, 2, 3 and 4.

## Results

The present study investigated *in vitro* developmental competence of bovine embryos obtained by two methods of various age, quality and developmental stage after two-step vitrification using increasing concentrations of cryoprotectants.

Table 1. *In vitro* development of bovine embryos (D 7-8; n = 67) obtained *in vitro* (method A) of different stages after vitrification

| Developmental stage of embryo | № of thawed embryos | № (%) of developed embryos | | |
|---|---|---|---|---|
| | | in culture | to hatching blastocyst | to hatched blastocyst |
| Morula | 10 | 3 (30) | 0 | 0 |
| Early blastocyst | 12 | 4 (33.3) | 1 (8.3) | 0 |
| Blastocyst | 18 | 6 (33.3) | 2 (11.1) | 1 (5.6) |
| Expanded blastocyst | 27 | 12 (44.4) | 3 (11.1) | 2 (7.4) |

In brackets, the percentage of embryos from the total of thawed embryos are shown.

Table 2. *In vitro* development of bovine embryos (D 7-8; n = 84) obtained *in vivo* (method B) of different stages after vitrification

| Developmental stage of embryo | № of thawed embryos | № (%) of developed embryos | | |
|---|---|---|---|---|
| | | in culture | to hatching blastocyst | to hatched blastocyst |
| Morula | 21 | 19 (90.5) | 10 (47.6) | 7 (33.3) |
| Early blastocyst | 15 | 14 (93.3) | 7 (46.6) | 4 (26.6) |
| Blastocyst | 22 | 18 (81.8) | 12 (54.5) | 6 (27.3) |
| Expanded blastocyst | 26 | 20 (76.9) | 10 (38.5) | 8 (30.8) |

In brackets, the percentage of embryos from the total of thawed embryos are shown.

Table 3. *In vitro* development of bovine embryos (D 7; n = 98) obtained *in vitro* (method A) of different quality after vitrification

| Developmental stage of embryo | № of thawed embryos | № (%) of developed embryos | | |
|---|---|---|---|---|
| | | in culture | to hatching blastocyst | to hatched blastocyst |
| 1 | 29 | 10 (34.5) | 3 (10.3) | 2 (6.9) |
| 2 | 30 | 9 (30) | 0 | 0 |
| 3 | 39 | 7 (17.9) | 0 | 0 |

In brackets, the percentage of embryos from the total of thawed embryos are shown.

Table 4. *In vitro* development of bovine embryos (D 7-8; n = 82) obtained *in vivo* (method B) of different quality after vitrification

| Developmental stage of embryo | № of thawed embryos | № (%) of developed embryos | | |
|---|---|---|---|---|
| | | in culture | to hatching blastocyst | to hatched blastocyst |
| 1 | 30 | 27 (90) | 16 (53.3) | 11 (36.6) |
| 2 | 32 | 24 (75) | 10 (31.3) | 7 (21.9) |
| 3 | 20 | 10 (50) | 4 (20) | 2 (10) |

In brackets, the percentage of embryos from the total of thawed embryos are shown.

When we analyzed the developmental stage of embryos produced by method A (Table 1) and method B (Table 2) we found significantly higher developmental competence of embryos retrieved by method B (4.5%; 3/67 vs. 29.8%; 25/84, $p < 0.001$). The hatching rates of embryos produced by method A and B and vitrified at morula stage were 0% (0/10) vs. 33.3% (7/21), $p > 0.05$. The corresponding proportions of developmental stages early blastocyst, blastocyst and expanded blastocyst were 0% (0/12) vs. 26.6% (4/15), 5.6% (1/18) vs. 27.3% (6/22), 7.4% (2/27) vs. 30.8% (8/26), respectively. In general, the hatched blastocyst stage was reached by 15.1% (50/331) of all thawed embryos produced by both methods A and B. When we compared the quality grade of embryos produced by method A (Table 3) and method B (Table 4) we found significantly higher developmental competence of embryos retrieved by method B (2.0% vs. 24.4%, $p < 0.001$). Furthermore, we noted a higher hatching rate at grade 1 quality embryos retrieved by method A and B (6.9%; 2/29 and 36.6%; 11/30, $p < 0.01$) compared to quality grade 2 (0%; 0/30 and 21.9%; 7/32, $p < 0.05$) and quality grade 3 (0%; 0/39 and 10%; 2/20, $p > 0.05$).

## Discussion

When slow freezing is used, it is difficult to completely eliminate injuries resulting from ice formation (Vajta et al. 1998). Furthermore, the slow freezing method requires a long period of time before embryos can be plunged into liquid nitrogen. To prevent the chilling injury during bovine embryo cryopreservation, it has been suggested that rapid cooling may be better than slow cooling (Pollard and Leibo 1994). Vitrification simplifies the cooling process, because embryos can be rapidly cooled directly in liquid nitrogen (Martino et al. 1996; Arav and Zeron 1997). Increasing the speed of thermal conduction and decreasing the toxicity of cryoprotectants is an ideal approach to embryo cryostorage by vitrification. However, the actual rate of heat transfer during vitrification may vary, depending on the device used and the movement of immersion. In addition, it is very important to mention that every cell has its own optimal cooling rate. Many studies provide information about the sensitivity of bovine embryos to cryopreservation (Vajta et al. 1997; 1998; 1999; Enright et al. 2000; Dobrinsky et al. 2002; Assumpcao et al. 2008) especially about the quality, developmental stage and origin of embryos, intracellular lipid content, culture conditions,

type or combination of cryoprotectants and method of cryopreservation and thawing. A crucial role is played by the size of the biological object. The smaller the sample, the higher is the chance of survival in the freezing process. In general, oocytes, zygotes and early stages of embryos are much more sensitive to chilling injuries than blastocysts and expanded blastocysts. A different situation exists with blastocysts after hatching, when their size increases and the chances of successful cryopreservation rapidly decrease. Mahmoudzadeh et al. (1995) demonstrated higher survival and hatching rates after two-step vitrification, especially at the blastocyst stage (75% and 38%, respectively) and the expanded blastocyst stage (89% and 69%, respectively). Similar data were obtained by Lopatarova et al. (2002) who achieved higher survival rate ($p < 0.05$) of embryos in the stage of expanded blastocyst cryopreserved by OPS (Open Pulled Straw) vitrification (38.6%; 27/70) compared to embryos cryopreserved by conventional freezing (34.5%; 29/84). Lazar et al. (2000) demonstrated that advanced stages of IVP embryos can be vitrified successfully with hatching rates reaching 60% to 94%. The authors in this study reached up to 81% re-expansion rate after 24 h of culture. We obtained analogous results at blastocyst and expanded blastocyst stage (see Table 1 and Table 2) compared to morula and early blastocyst stage which did not proceed. The morphological quality of embryos is one of the most important factors. In general, a lower morphological quality is connected with a lower survival rate, as mentioned in our study.

In these embryos, a higher content of damaged blastomeres of unknown origin was found. The cause could be the formation of intracellular ice crystals due to incomplete cellular dehydration, decreased membrane permeability or inappropriate conditions of thawing.

Our findings indicate that vitrification method is a good alternative to conventional freezing because it results in high survival and *in vitro* development rates of bovine embryos. Many authors had described analogous findings (Dynnies et al. 1996; Vajta et al. 1997; Lieberman and Tucker 2006; Assumpcao et al. 2008; Pereira and Marques 2008). On the basis of our results, it can be concluded that two-step vitrification can accelerate, simplify and reduce the financial costs of the cryopreservation method of *in vitro* produced bovine embryos.

In conclusion, it is well known that cryopreservation is a very stressful procedure, especially for *in vitro* produced embryos (Leibo et al. 1996; Vajta et al. 1998). For the future, it is necessary to establish a standardized vitrification protocol in both veterinary and human medicine that would be applicable to cryopreservation of different developmental stages and origins. Further research should focus on enhancement of embryo resistance and improvement of *in vitro* culture conditions, thus rendering the cryopreservation procedure more effective.

## Vliv dvoustupňové vitrifikace na vývojovou kompetenci bovinních embryí získaných *in vitro* a *in vivo*

Cílem této práce bylo stanovit vliv dvoustupňové vitrifikace na přežívání embryí Holštýnsko-Fríského skotu získaných metodou produkce *in vitro* (metoda A) či *in vivo* (metoda B). Embrya vhodná k vitrifikaci byla dvoustupňově zmrazena, přičemž byla použita rostoucí koncentrace dimethylsulfoxidu (DMSO) a ethylenglykolu (EG). Po rozmrazení byla zhodnocena kvalita a vývojové stádium embryí. Vývojová kompetence embryí různé kvality získaných metodou B (n = 82) byla ve srovnání s embryi získanými metodou A (n = 98) signifikantně vyšší ($p < 0{,}001$). Nejlepších výsledků jsme dosáhli při vitrifikaci embryí kvality 1; konkrétně bylo u metody A dosaženo 6.9% (2/29) stádií vyklubaných blastocyst a u metody B 36.7% (11/30) stádií vyklubaných blastocyst ($p < 0.01$). Co se týká vývojové kompetence embryí různého stádia vývoje, dosáhli jsme signifikantně lepších výsledků ($p < 0.001$) při vitrifikaci embryí získaných metodou B (n = 84) než tomu bylo

u embryí produkovaných metodou A (n = 67). Vyššího počtu vyklubaných embryí jsme dosáhli při vitrifikaci stádií expandovaných blastocyst; konkrétně dosáhlo stádia vyklubané blastocysty 7.4% (2/27) embryí získaných metodou A a 30.8% (8/26) embryí získaných metodou B ($p < 0.05$). V obecném měřítku jsme dosáhli 15.1% (50/331) vyklubaných blastocyst ze všech rozmrazených embryí získaných metodou A a B.

Na závěr lze říci, že při použití metody dvoustupňové vitrifikace na embrya stupně kvality 1 ve stádiu expandované blastocysty produkované *in vitro* či stádiu moruly produkované *in vivo* jsme dosáhli nejvyššího stupně vyklubání embryí.

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