

Short note

## Effect of post-thaw cell rehydration at 4 °C on survival of frozen and vitrified IVP-derived bovine embryos

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**Abstract** — In vitro-produced bovine embryos (IVP) were either frozen in 10 % glycerol in a phosphate-buffered saline solution (PBS) using conventional slow freezing or vitrified in 25 % glycerol and 25 % ethylene glycol in PBS. The results of viability and hatching rates were compared between frozen and vitrified embryos after thawing and dilution using one of three different protocols: (A) a three-step dilution procedure, (B) a one-step dilution procedure or (C) a procedure in which embryos were kept in situ inside the straw at 4 °C for 10 min during a one-step dilution procedure. No significant differences in embryo survival were found among protocols A, B and C for frozen embryos and between protocols A and B for vitrified embryos. Viability and hatching rates of vitrified embryos thawed and diluted by protocol C (73 and 62 %) were significantly enhanced ( $P < 0.05$ ) in comparison to those obtained with protocol A (55 and 41.6 %) or protocol B (54.5 and 35.3 %). These results indicate that for vitrified IVP bovine embryos, direct in-straw rehydration at 4 °C for 10 min improves embryo survival and it could be a practical procedure for use under field conditions where there is sometimes a longer interval between thawing and transfer. © Inra/Elsevier, Paris.

**IVP / bovine / embryos / rehydration / vitrification / survival**

**Résumé** — L'effet de la réhydratation cellulaire après décongélation à 4 °C sur la survie des embryons bovins PIV, congelés et vitrifiés. Des embryons bovins produits in vitro (PIV) ont été soit congelés dans 10 % glycérol dans une solution saline tamponnée avec du phosphate (STP) utilisant la congélation lente conventionnelle soit vitrifiés dans 25 % glycérol et 25 % éthylène glycol dans STP. Les résultats de viabilité et les vitesses d'éclosion ont été comparés pour les embryons congelés et ceux vitrifiés après décongélation et dilution utilisant un des trois protocoles : (A) une procédure de dilution à trois étapes, (B) une procédure de dilution à une seule étape, et (C) une procédure où les embryons sont gardés in situ à l'intérieur de la paille à 4 °C pendant 10 min au cours d'une procédure de dilution à une étape. Aucune différence significative de survie des embryons n'a été trouvée pour les protocoles A, B et C des embryons congelés et pour les protocoles A et B des embryons vitrifiés. La viabilité et la vitesse d'éclosion des embryons vitrifiés décongelés et dilués par le protocole C (73 et 62 %) ont été augmentées significativement ( $p < 0,05$ ) en comparaison à celles obtenues avec le protocole A (55 et 41,6 %) ou le protocole B (54,5 et 35,3 %). Ces résultats indiquent que pour les

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embryons bovins vitrifiés PIV, la réhydratation directe dans la paille à 4 °C pendant 10 min améliore la survie des embryons et il pourrait être une procédure pratique pour l'utilisation dans des conditions de terrain où il y a parfois un plus long intervalle entre la décongélation et le transfert. © Inra/Elsevier, Paris.

### **PIV / bovin / embryons / post-décongélation / réhydratation**

The efficient utilization of in vitro-produced bovine embryos (IVP) requires their successful cryopreservation. In the context of freezing, IVP bovine embryos differ considerably from those produced in vivo, showing an increased sensitivity to chilling and freezing [6]. The vitrification procedure is a simplification of the freezing protocol and it yields a high survival of IVP bovine embryos after thawing [5]. The survival rates of frozen or vitrified IVP bovine embryos, measured as post-thaw development in culture, are, however, lower than those reported for in vivo-derived embryos [9].

Direct transfer of cryopreserved-thawed embryos reduces the time and expense involved in embryo transfer procedures and it offers handling advantages over conventional freezing procedures, which require step-wise removal of the cryoprotectant after thawing [10, 11]. Under field conditions, however, there is sometimes a long time delay between thawing the embryo and its transfer into a recipient. This can have deleterious effects due to the high toxicity of cryoprotectants. We wanted to simplify the thawing procedure in order to facilitate transport and application in field conditions. The aim of this work was to determine the effect of post-thaw cell rehydration at 4 °C on the survival of cryopreserved embryos. For this purpose, we have compared three rehydration protocols on the survival and developmental capacity of frozen and vitrified IVP bovine embryos.

Details of in vitro production and culture of bovine embryos are available elsewhere [2]. Overall, the development from the one cell to the blastocyst stage was 20–30 % in in vitro culture. Only good quality embryos, which had developed into blastocysts on days 7 and 8 after insemination, were used

for these experiments. Embryos from each experimental pool were randomly allocated to all treatment groups.

In all experiments, among the six treatment groups, cryoprotectants were dissolved in phosphate-buffered saline (PBS) supplemented with 20 % foetal calf serum (FCS). Embryos were frozen in 10 % glycerol in PBS using a conventional freezing method [8]. Briefly, embryos were equilibrated in 10 % glycerol (Gly) for 10 min at room temperature (20–23 °C); during this time they were loaded into 0.25 mL straws. The straws (Minitüb, Germany) were placed directly into a pre-cooled alcohol bath of a programmable freezer at –7 °C (Bio-Cool, FTS Systems, New York, USA). After 2 min, the straws were seeded and maintained at –7 °C for another 8 min. Then, they were cooled to –35 °C at a rate of 0.5 °C·min<sup>-1</sup> before being plunged into liquid nitrogen (LN<sub>2</sub>).

For vitrification, embryos were put into a 10 % Gly solution for 5 min at room temperature (17–20 °C). Afterwards, they were transferred into a 10 % Gly + 20 % ethylene glycol (EG) solution for 5 min; and finally, the embryos were placed in the vitrification solution containing 25 % Gly and 25 % EG for 30 s, and they were introduced into 0.25 mL plastic straws and plunged into LN<sub>2</sub> for storage. Those embryos assigned to the in situ one-step dilution were placed in the straw in one drop (50 µL) of vitrification solution flanked by two columns of a 0.5 M sucrose solution (160 µL). Drops were separated by air bubbles.

After 15–20 days of storage in liquid nitrogen, the straws were placed in air for 5–10 s and plunged into a 30 °C water bath for 20 s. After thawing, the cryoprotectant was removed by one of three treatments:

(A) a three-step dilution procedure, (B) a one-step dilution procedure and (C) a direct in-straw one-step dilution at 4 °C for 10 min. In the three-step dilution procedure, the cryoprotectant was removed in three steps of decreasing concentrations (50, 25 and 0 % of initial cryoprotectant concentration) in PBS supplemented with 0.3 M sucrose and 10 % FCS (5 min per step) at room temperature. In the one-step procedure, the content of each straw was transferred into a Petri dish with a 0.5 M sucrose solution for 5 min. In the in situ one-step dilution procedure, the straw was shaken three times to mix the liquid columns containing 0.5 M sucrose and the embryo with the vitrification solution. Subsequently, each straw was placed horizontally (to prevent embryo sedimentation and migration to the end of the straw) on the surface of ice for 10 min.

After cryoprotectant removal, the embryos were transferred into a Petri dish, washed with PBS and cultured in vitro in a modified SOF medium (synthetic oviductal fluid medium) [2] supplemented with 10 % FCS medium for 3 days. Embryo appearance was evaluated morphologically once immediately after warming (viability) and the hatching rate was recorded visually after culture in mSOF + 10 % FCS medium for 2–3 days. The differences between the treatments were determined by  $\chi^2$  analysis with the Yates correction.

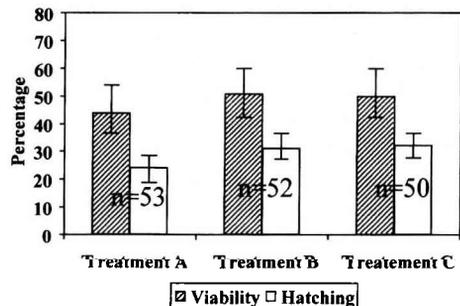
For IVP bovine embryos there is no simple protocol for cryopreservation. In this study, freezing and vitrification of IVP bovine embryos were performed using standard protocols reported to yield good embryo survival results. The use of Gly as a permeable cryoprotectant has produced high pregnancy rates [3, 7]; therefore, Gly was selected for our experiment. For the vitrification protocol we have selected a solution consisting of 25 % Gly and 25 % EG. This solution also allowed high in vitro survival rates and high pregnancy rates [1].

Overall, survival rates were higher upon vitrification than upon slow cooling. This agrees with other authors who have

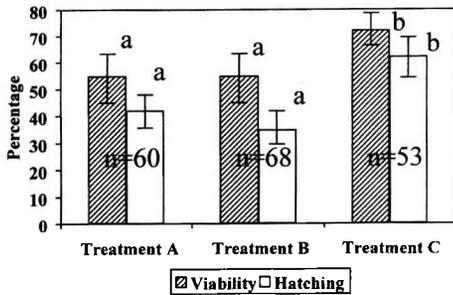
described that vitrification seems to be superior to slow freezing since it better overcomes the increased chilling sensitivity of in vitro-derived embryos [9].

Embryo viability and hatching rates of IVP bovine embryos frozen in 10 % Gly did not vary among thawing procedures (figure 1). However, with vitrified embryos, the in situ one-step removal at 4 °C improved both viability and hatching rates significantly (72 and 62 % respectively;  $P < 0.05$ ) in comparison to one-step removal (54.5 and 35 %) or three-step removal (55 and 41 %) (figure 2,  $P < 0.05$ ).

Under field conditions, the time between thawing and transfer to the recipient is sometimes long, allowing the embryos to be in contact with the cryoprotectant during a non-desirable period. It has been suggested that during the dilution of permeable cryoprotectants the process of post-thaw cell rehydration is more important for the viability of embryos than the chemical toxicity of cryoprotectants [4]. Sucrose facilitates the removal of intracellular cryoprotectants during dilution and reduces chemical toxicity of cryoprotectants by causing embryos to shrink rapidly and by maintaining a high osmotic pressure in the extracellular medium. At 4 °C, the cryoprotectant per-



**Figure 1.** Viability and hatching rates of in vitro-produced bovine blastocysts following freezing in glycerol and thawing in three different treatments: (A) a three-step dilution procedure, (B) a one-step dilution procedure and (C) a procedure in which embryos were kept in situ inside the straw at 4 °C for 10 min during a one-step dilution procedure. Data represent means  $\pm$  SD of three or more replicates. Numbers inside the bars are the numbers of embryos in each group.



**Figure 2.** Viability and hatching rates of in vitro-produced bovine blastocysts following vitrification in glycerol and ethylene glycol and thawed in three different treatments: (A) a three-step dilution procedure, (B) a one-step dilution procedure and (C) a procedure in which embryos were kept in situ inside the straw at 4 °C for 10 min during a one-step dilution procedure. Data represent means  $\pm$  SD of three or more replicates (ab,  $P < 0.05$ , Chi-square test). Numbers inside the bars are the numbers of embryos in each group.

meability is low; meanwhile, with embryos that were transferred at room temperature, the high temperature might increase the cryoprotectant permeability of the embryo membrane to a toxic level (the shrinkage process is faster at room temperature than at 4 °C). Therefore, the procedure may increase the damage to embryonic cells produced by a fast and/or excessive rehydration process.

The procedure of in situ one-step removal at 4 °C is superior than the procedures of one-step or three-step removal for vitrified embryos, but not for slowly frozen embryos. We think that slowly frozen embryos shrink upon dilution in the presence of sucrose (the volume after thawing is about 105 %, and after 0.5 M sucrose addition is 40–50 %), whereas vitrified embryos undergo a transient swelling before returning to a lower volume (after thawing, it has a volume of 40 %, swelling to approximately 60 %, then returning to 35 % upon 0.5 M sucrose dilution). For slowly frozen embryos, it is therefore not the rehydration process that can harm the cells, but rather dehydration.

Further work is required to examine the details of changes at the ultrastructural level that are caused by the slow post-thaw cell

rehydration process. However, these initial experiments have established a simple post-thaw dilution of permeable cryoprotectants that can be applicable under field conditions.

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