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Effect of vitrification technique and assisted hatching on rabbit embryo developmental rate

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Summary

The aim of the study was to determine the efficiency of two vitrification techniques followed by two assisted hatching (AH) techniques based on post-thaw developmental capacity of precompacted rabbit embryos and their ability to leave the zona pellucida (hatching) during *in vitro* culture. The total cell number and embryo diameter as additional markers of embryo quality after warming were evaluated. *In vivo* fertilized, *in vitro* cultured 8–12-cell rabbit embryos obtained from superovulated rabbit does were cryopreserved by two-step vitrification method using ethylene glycol (EG) as cryoprotectant or by one-step vitrification method with EG and Ficoll (EG + Ficoll). Thawed embryos were subjected to enzymatic or mechanical AH. Vitrified EG group showed significantly lower ($P < 0.05$) blastocyst rate (22.5%) and hatching rate (15%) than those vitrified with EG + Ficoll (63 and 63% resp.) and that of control (97 and 97% respectively). Significantly lower values of total cell number ($P < 0.05$) as well as embryo diameter ($P < 0.01$) in EG group compared with EG + Ficoll and control group were recorded. No significant difference was found in developmental potential of warmed embryos treated by either mechanical or enzymatic AH. The present study demonstrates that the EG + Ficoll vitrification protocol provides superior embryo survival rates over the EG vitrification protocol for 8–12-cell stage precompacted rabbit embryos. No positive effect of either mechanical or enzymatic AH on the post-thaw viability and quality of rabbit embryos *in vitro* was observed.

Keywords: Assisted hatching, Cell number, Embryo diameter, Rabbit embryo, Vitrification

Introduction

Vitrification as an embryo freezing method is successfully used for cryopreservation of embryos of different animal species as well as humans. For human embryos vitrification has been shown to be more favourable than slow freezing with regard to survival, ongoing pregnancy and implantation rates (Kuleshova & Lopata, 2002; Stehlik *et al.*, 2005). The survival rate after vitrification differs depending on the method of cryopreservation (Zhao *et al.*, 2007), number of steps, cooling rates (Hredzák *et al.*, 2006), type and concentration of cryoprotective agent (CPA) (Zhou *et al.*, 2007), developmental stage of embryo (Zhou *et al.*, 2005) as well as the embryo quality alone (Fabian *et al.*, 2005).

Several literature sources reported unfavourable ZP hardening as a possible reason of lower developmental potential of frozen/thawed embryos to hatching stage (Vanderzwalmen *et al.*, 2003). For this reason, different types of assisted hatching (AH) have been developed.

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AH can be performed by total mechanical and chemical penetration of ZP or partial enzymatic thinning of ZP by pronase enzyme or laser (Blake *et al.*, 2001). A study comparing these four different techniques showed similar implantation and pregnancy rates (Balaban *et al.*, 2002).

Other non-invasive parameters for the testing of embryo viability seems to be the embryo diameter and total cell number (Mori *et al.*, 2002; Makarevich *et al.*, 2006). The measurement of embryo diameter and counting of cell number using nuclear staining with vital permanent dyes as Hoechst 33342 would enable the non-invasive selection of the embryos with best developmental potential without its destruction.

The present study aimed to compare directly: (1) the effectiveness of two vitrification procedures; and (2) the influence of mechanical and enzymatic assisted hatching techniques applied after embryo warming on the *in vitro* development, cell count and diameter of *in vivo* fertilized, *in vitro* cultured precompacted rabbit embryos.

Materials and methods

Unless otherwise specified, chemicals were obtained from Sigma.

Recovery of rabbit embryos

Female New Zealand white rabbit does reared on the local farm were treated with PMSG (Werfaser, Alvetra and WERFF) i.m. at 20 IU/kg live weight, 72 h before mating. Immediately prior to mating, the females were injected i.m. with hCG (Werfachor, Alvetra und WERFFT) at 40 IU/kg live weight, afterwards the females were mated with a male of proven fertility from the same breed. The pronuclear stage eggs were flushed from the oviduct of slaughtered animals with phosphate-buffered saline (PBS, Gibco-Life Technologies) 19 to 20 h post coitum (hpc). The flushed eggs were evaluated morphologically and the eggs with both pronuclei were selected and cultured additionally for 24 h *in vitro* in a culture medium (k-DMEM, Gibco BRL) supplemented with 10% fetal calf serum (FCS, Gibco BRL.) in a humidified atmosphere of 5% CO_2 at 39 °C.

Vitrification procedures

To avoid individual variations between does, the zygotes recovered from five does were used in five consecutive experiments. *In vivo* fertilized, *in vitro* cultured rabbit embryos at 8–12-cell stages were vitrified using one of the two procedures. In first procedure, a two-step cryoprotectant loading process was used. The vitrification solution (VS) composed of 40% ethylene glycol (EG) and 1 mol/l sucrose in the basic medium (HEPES-buffered CIM, Gibco BRL supplemented with 20% FCS). The precompacted embryos were then exposed to VS for 2 min and 30 s, respectively. In second procedure, an one-step loading process in the VS containing 40% EG, 18% Ficoll 70 and 0.3 mol/l sucrose (Papis *et al.*, 2005) dissolved in a basic medium was used. Vitrification solutions were equilibrated by prewarming at an ambient temperature. In both cases a group of embryos (maximum five) was put inside a French straw in a small volume of medium (0.5 µl) and directly plunged into liquid nitrogen. The straws were stored in liquid nitrogen for 1–3 days.

Warming process of vitrification and artificially assisted hatching (AH)

EG vitrified embryos were warmed using two-step dilution in sucrose solution. The embryos were expelled and plunged into 1 and 0.5 mol/l sucrose solution prepared in a basic medium, 5 and 5 min, respectively. EG + Ficoll vitrified embryos were directly expelled into 0.5 mol/l sucrose solution and equilibrated for 8 min. Immediately after warming, embryos were divided into two groups for either mechanical or enzymatic AH. For mechanical AH the zona pellucida of the embryo immobilized on a holding pipette was penetrated with a microinjection needle with a diameter of approximately 15 µm to make a single-holed zona puncture. Enzymatic AH was done by short (30 s) exposure of embryos to 0.5% (v/v) pronase solution in PBS. Developmental capacity of thawed embryos was assessed on the basis of their ability to leave the zona pellucida (hatching) after 3 days of *in vitro* culture in k-DMEM medium (Gibco BRL.) + 10% FCS in a humidified atmosphere of 5% CO_2 at 39 °C.

Determination of cell count and diameter of rabbit embryos

Total cell number of blastocysts was determined using Hoechst 33342 staining and counting under a Leica fluorescence microscope, as was previously described (Chrenek *et al.*, 2005). Embryo diameters, excepting ZP, were measured from the same images on the screen of the monitor using scale bar micrometer, which was previously calibrated on a ×40 objective and ×10 eye-pieces (Makarevich *et al.*, 2006).

Statistical analysis

Chi-squared test was used to determine differences between two vitrification procedures as well as between the percentage of embryos developing

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Table 1 Effect of vitrification method on the development, cell number and diameter (μm) of rabbit embryos

Group of embryos	No. embryos	No. vitrified/ warmed	No. blastocysts, *n* (%)*	No. hatching blastocysts *n* (%)*	Total cell number (± S.E.M.)**	Embryo diameter (± S.E.M.)**
EG	80	80/80	18 (22.5)[a]	12 (15)[a]	109.7 ± 2.73[c]	128.0 ± 1.67[e]
EG + Ficoll	60	60/60	38 (63)[b]	38 (63)[b]	118.0 ± 4.69[d]	134.2 ± 5.59[e,f]
Intact	72	–	70 (97)[c]	70 (97)[c]	121.0 ± 3.54[d]	141.0 ± 3.08[f]

*$P < 0.05$ (chi-squared test, the values within the column differ significantly); **$P < 0.05$, (one–way ANOVA, Kruskal–Wallis test). Numbers in columns with different superscripts differ significantly.

Table 2 Effect of assisted hatching (AH) on the development, cell number and diameter (μm) of rabbit embryos

Group of embryos	Method of vitrification	*N*	No. blastocysts, *n* (%)*	No. hatching blastocysts *n* (%)*	Total cell number (± S.E.M.)**	Embryo diameter (± S.E.M.)**
Control (no AH)	EG	80/80	18 (22.5)	12 (15)	109.7 ± 2.73	128.0 ± 1.67
AH (mechanical)	EG	55	11 (20)[a]	8 (13)[a]	109.5 ± 1.41[d]	118.0 ± 1.82[d]
AH (enzymatic)	EG	50	10 (20)[a]	10 (20)[b]	125.0 ± 2.94[e]	140.0 ± 6.16[e]
Control (no AH)	EG + Ficoll	60/60	38 (63)	38 (63)	118.0 ± 4.69	134.2 ± 5.59
AH (mechanical)	EG + Ficoll	54	45 (81)[b]	45 (81)[c]	111.0 ± 0.82[d]	125.0 ± 1.63[f]
AH (enzymatic)	EG + Ficoll	48	35 (72)[b]	35 (72)[c]	115.0 ± 1.47[f]	138.8 ± 2.63[e]

*$P < 0.01$ (chi-squared test), **$P < 0.05$ (one–way ANOVA, Kruskal–Wallis test). Numbers in columns with different superscripts differ significantly.

to blastocysts and hatching blastocysts stage after mechanical or enzymatic AH. Data for embryo cell counts and diameters were expressed as mean values ± S.E.M. and assessed by analysis of variance (ANOVA) using Kruskal–Wallis test. Differences between groups at $P < 0.05$ were considered as significant.

Results

Effect of vitrification technique on the development, cell number and diameter of rabbit embryos

Table 1 summarizes survival and quality characteristics of rabbit embryos subjected to either of two procedures of vitrification at 8–12-cell stage. Blastocyst rate (22.5%) and hatching rate (15%) in the vitrified EG group were significantly lower than that for the vitrified EG + Ficoll group (63 and 63% resp.) and intact non-vitrified group (97 and 97% respectively). Total cell number was significantly lower ($P < 0.05$) in EG vitrified embryos compared to EG + Ficoll and the intact group. Embryo diameter was lower ($P < 0.01$) in EG vitrified group compared to the intact ones, whilst no difference was observed between EG + Ficoll vitrified and intact embryos.

Effect of assisted hatching (AH) on the development, cell number and diameter of rabbit embryos

Developmental rates for vitrified-warmed rabbit embryos following mechanical or enzymatic assisted hatching are compared (Table 2). In EG group with enzymatic AH and in both EG + Ficoll groups all blastocyst stage embryos (100%) reached hatching stage. However, in EG group with mechanical AH from 11 blastocysts only eight reached hatching stage. Total cell number in both vitrification groups (EG and EG + Ficoll), was lower ($P < 0.01$) when mechanical AH was applied compared to enzymatic AH. In embryo diameter significant ($P < 0.05$) differences were observed between mechanical and enzymatic AH using both vitrification procedures.

Discussion

Since the first cryopreservation study in rabbit, the best embryo survival rates have been obtained at the morula stage (Vicente *et al.*, 2003). This embryo stage allows the conventional freezing procedures to be substituted efficiently by a simple method of vitrification (Naik *et al.*, 2005). Usually at *in vivo* experiments rabbit embryos after microinjection are cultured for further 24 h and then are transferred to recipients at the 8-cell stage. It is proper to use mucin coat-free embryos for micromanipulation only, which corresponds to 19–20 hpc. Following manipulations, mucin coat may be restored by transfer to the oviduct of synchronized recipients to increase their implantation potential. Transfer of *in vitro* manipulated and *in vitro* cultured embryos at higher stages than the blastocyst does not give satisfactory implantation rates. For this reason rabbit micro-manipulated embryos at 8-cell stage are more proper for the cryopreservation and

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embryo bank establishment. Precompacted (1- to 8-cell stage) embryos are more sensitive to external influences such as cryopreservation than post-compacted and differentiated embryos. This stage-dependent viability of vitrified rabbit embryos was reported in an earlier report (Smorag *et al.*, 1989). For this reason a choice of effective vitrification procedure with high post-thaw survival rates is very important for the embryo bank foundation.

Important factors affecting success of vitrification include rates of temperature decrease and cooling of vitrification solutions. Hredzák *et al.* (2006) observed a decrease in hatching rate of blastocysts produced from 8-cell mouse embryos vitrified in straws (8%) compared to the group frozen in pipetting tips (38%) or introduced dropwise directly into liquid nitrogen (60%). An important role of cooling rates of the solution is ascribed to the freezing container, thermal conductivity of its walls and the volume of vitrification solution (Liebermann *et al.*, 2003). We used, as a carrier system, classical French straw inside which we put the group of embryos at a minimum volume (<1.0 µl) to meet this requirement. The embryos adhered to the straw wall during the cooling process and were expelled to the thawing medium without drop-outs.

Penetrating cryoprotectants such as propylene glycol or ethylene glycol that require the highest concentrations to vitrify and displace the most water are actually the least toxic. In our study the vitrification solution based on ethylene glycol at concentration of 40% (v/v) in basic medium had inferior effect on post-thaw viability of rabbit precompacted embryos. The addition of macromolecular substance Ficoll (mol. wt 70,000) to vitrification medium improved the survival and blastocyst rate of vitrified/warmed embryos to 67% in comparison with 22.5% in the EG group. This polymer is less toxic and can protect embryos against cryoinjury by mitigating the mechanical stress occurring during cryopreservation, and by building a viscous matrix around these embryos, which prevents crystallization during cooling and warming (Kuleshova *et al.*, 2001). Vicente & Garcia-Ximénez (1994) observed worst survival rate of the cryopreserved rabbit morulae and development to blastocysts *in vitro* in the EG-treated embryos compared to mixture of ethylene glycol and dimethylsulfoxide (EG + DMSO, 20% v/v).

Hardening of ZP may occur naturally following fertilization in order to block polyspermic fertilization, after prolonged exposure of embryos to artificial culture conditions and after cryopreservation (De Vos & Van Steirteghem, 2000). Popelková *et al.* (2005) observed high significant differences in hatching rates between OPS vitrified rabbit embryos (56%) and non-frozen ones (94%). This decrease in the hatching rate may reflect altered viability of the thawed embryos or structural changes of the zona. Studies specifically addressed the issue of AH performed on frozen–thawed embryos reported controversial results. Kong *et al.* (2000) declared that enzyme-assisted hatching following vitrification enhances post-thaw hatching rates of bovine *in vitro* produced embryos. Ng *et al.* (2005) compared implantation and pregnancy rates of frozen–thawed embryo transfer (FET) cycles with and without laser AH and concluded that laser AH does not improve the implantation rate of FET cycles. Balaban *et al.* (2002) found similar implantation and pregnancy rates for four different techniques of AH including acidic Tyrode's solution, partial zona dissection, diode laser and pronase zona thinning. In the present study, the beneficial effect of total mechanical ZP breaching as well as enzymatic ZP thinning AH after vitrification was not evident as no significant increase in the blastocyst hatching rate during post–warm *in vitro* culture was recorded. The study of Mantoudis *et al.* (2001) and Blake *et al.* (2001) showed a higher hatching rate when AH was performed by zona thinning rather than by complete zona breaching. These studies suggest that the opening the zona pellucida may have adverse effects, such as abnormal blastocyst expansion, loss of blastomeres through the breached zona and lack of the protective effect of the ZP against infectious agents. In our *in vitro* study both techniques of AH had no adverse effect on the further development of rabbit embryos in culture.

Makarevich *et al.* (2006) tested the effect of microinjection (Mi) of foreign gene into rabbit egg pronucleus and epidermal growth factor (EGF) addition on the blastocyst rate, total cell number and the diameter of embryos. The microinjection procedure in their study decreased blastocyst rate and cell number, but did not affect the embryo diameter. In our study the embryos vitrified by EG had lower total cell number and embryo diameter compared to intact non-vitrified ones, and also showed lower total cell number compared with EG + Ficoll group. The EG vitrified group showed also significantly lower blastocyst and hatching rate. Most of embryos did not develop further after thawing.

In conclusion, the one-step vitrification procedure with EG and Ficoll used as cryoprotective agents gave superior results of post-thaw developmental potential of precompacted stage rabbit embryos cultured *in vitro*. The two-step vitrification with EG based solution showed to be less effective in terms of hatching rate and quality of embryos (cell number and embryo diameter). No influence of either mechanical or enzyme-assisted hatching on the post-thaw viability and quality of rabbit embryos *in vitro* was observed.

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References

Balaban, B., Urman, B., Alatas, C., Mercan, R., Mumcu, A. & Isiklar, A. (2002). A comparison of four different techniques of assisted hatching. *Hum. Reprod.* **17**, 1239–43.

Blake, D.A., Forsberg, A.S., Johansson, B.R. & Wikland, M. (2001). Laser zona pellucida thinning – an alternative approach to assisted hatching. *Hum. Reprod.* **16**, 1959–64.

Chrenek, P. & Makarevich, A. (2005). Production of rabbit chimeric embryos by aggregation of zona-free nuclear transfer blastomeres. *Zygote* **13**, 39–44.

De Vos, A. & Van Steirteghem, A. (2000). Zona hardening, zona drilling and assisted hatching: new achievements in assisted reproduction. *Cells Tissues Organs* **166**, 220–7.

Fabian, D., Gjorret, J.O., Berthelot, F., Martinat-Botté, F. & Maddox-Hytel, P. (2005). Ultrastructure and cell death of *in vivo* derived and vitrified porcine blastocysts. *Mol. Reprod. Dev.* **70**(2), 155–65.

Hredzák, R., Ostró, A., Ždilová, V., Maraček, I. & Kačmárik, J. (2006). Survival of mouse embryos after vitrification depending on the cooling rate of the cryoprotectant solution. *Acta Veterinaria Hungarica* **54**(1), 117–25.

Kaidi, S., Bernard, S., Lambert, P., Massip, A., Dessy, F. & Donnay, I. (2001). Effect of conventional controlled-rate freezing and vitrification on morphology and metabolism of bovine blastocysts produced *in vitro*. *Biol. Reprod.* **65**, 1127–34.

Kong, I.K., Lee, S.I., Im, Y.T., Cho, S.G., Ohh, H.J., Ohh, D.H. & Bae, I.H. (2000). Improvement of post-thaw hatching rates of in vitro-produced bovine embryos vitrified by ultra-mini straw (abstract). *Theriogenology* **53**, 258.

Kuleshova, L.L., Shaw, J.M. & Trounson, A.O. (2001). Studies on replacing most of the penetrating cryoprotectant by polymers for embryo cryopreservation. *Cryobiology* **43**, 21–31.

Kuleshova, L.L. & Lopata, A. (2002). Vitrification can be more favourable than slow cooling. *Fertil. Steril.* **78**, 449–54.

Liebermann, J., Dietl, J. & Vanderzwalmen, P. (2003). Recent developments in human oocyte, embryo and blastocyst vitrification: where are we now? *Reprod. Biomed. Online* **7**, 623–33.

Makarevich, A.V., Chrenek, P. & Fl'ak, P. (2006). The influence of microinjection of foreign gene into the pronucleus of fertilized egg on the preimplantation development, cell number and diameter of rabbit embryos. *Asian-Aust. J. Anim. Sci.* **19**(2), 171–5.

Mori, M., Otoi, T. & Suzuki, T. (2002). Correlation between the cell number and diameter in bovine embryos produced *in vitro*. *Reprod. Dom. Anim.* **37**, 181–4.

Naik, B.R., Rao, B.S., Vagdevi, R., Gnanprakash, M., Amarnath, D. & Rao, V.H. (2005). Conventional slow freezing, vitrification and open pulled straw (OPS) vitrification of rabbit embryos. *Anim. Reprod. Sci.* **86**, 329–38.

Ng, E.H.Y., Naveed, F., Lau, E.Y.L., Yeung, W.S.B., Chan, C.Ch.W., Tang, O.S. & Ho, P.Ch. (2005). A randomized double-blind controlled study of the efficacy of laser-assisted hatching on implantation and pregnancy rates of frozen–thawed embryo transfer at the cleavage stage. *Hum. Reprod.* **20**(4), 979–85.

Papis, K., Sypecka, J., Korwin-Kossakowski, M., Wenta-Muchalska, E., Bilska, B. (2005). Banking of embryos of mutated, paralytic tremor rabbit by means of vitrification. *Laboratory Animals* **39**, 284–9.

Popelková, M., Chrenek, P., Pivko, J., Makarevich, A.V., Kubovičová, E. & Kačmárik, J. (2005). Survival and ultrastructure of gene-microinjected rabbit embryos after vitrification. *Zygote* **13**, 283–93.

Smorag, Z., Gajda, B., Wieczorek, B. & Jura, J. (1989). Stage dependent viability of vitrified rabbit embryos. *Theriogenology* **31**, 1227–31.

Stehlik, E., Stehlik, J., Katayama, K.P., Kuwayama, M., Jambor, V., Brohammer, R. & Kato, O. (2005). Vitrification demonstrates significant improvement versus slow freezing of human blastocysts. *Reprod. Biomed. Online* **11**, 53–7.

Vanderzwalmen, P., Bertin, G., Debauche, Ch., Standaert, V., Bollen, N., Van Roosendaal, E., Vandervorst, M., Schoysman, R. & Zech, N. (2003). Vitrification of human blastocysts with Hemi-Straw carrier: application of assisted hatching after thawing. *Hum. Reprod.* **18**, 1504–11.

Vicente, J.S. & Garcia-Ximénez, F. (1994). Osmotic and cryoprotective effects of a mixture of DMSO and ethylene glycol on rabbit morulae. *Theriogenology* **42**, 1205–15.

Vicente, J.S., Viudes-De-Castro, M.P., Garcia, M.L. & Baselga, M. (2003). Effect of rabbit line on a program of cryopreserved embryos by vitrification. *Reprod. Nutr. Dev.* **43**, 137–43.

Zhao, X.M., Quan, G.B., Zhou, G.B., Hou, Y.P. & Zhu, S.E. (2007). Conventional freezing, straw, and open-pulled straw vitrification of mouse two pronuclear (2-PN) embryos. *Anim. Biotechnol.* **18**, 203–12.

Zhou, G.B., Hou, Y.P., Jin, F., Yang, Q.E., Yang, Z.Q., Quan, G.B., Tan, H.M. & Zhu, S.E. (2005). Vitrification of mouse embryos at various stages by open-pulled straw (OPS) method. *Anim. Biotechnol.* **16**, 153–63.

Zhou, C., Zhou, G.B., Zhu, S.E., Hou, Y.P., Jin, F., Zhao, X.M. & Hong, Q.H. (2007). Open-pulled (OPS) vitrification of mouse hatched blastocysts. *Anim. Biotechnol.* **18**, 45–54.