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Post-thaw culture in presence of insulin-like growth factor I improves the quality of cattle cryopreserved embryos

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Summary

The goal of this study was to examine the effect of insulin-like growth factor I (IGF-I; added during post-thaw culture (48 h)) on the preimplantation viability and quality of cryopreserved bovine *in vivo* recovered embryos. The morula stage embryos, non-surgically recovered from superovulated dairy cows of Czech Fleckvieh cattle breed, had previously been cryopreserved by a slow freezing technique and stored in liquid nitrogen since 1989–1990. Following thawing, the embryos were cultured for 48 h either alone (no IGF-I) or in the presence of IGF-I (10 or 100 ng/ml); non-cultured embryos served as a control. Thereafter, the embryos were analyzed for cleavage to the blastocyst stage, apoptosis (TUNEL), embryo cell number and quality of actin cytoskeleton. Following post-thaw culture 41% of embryos developed to advanced blastocysts. IGF-I increased this per cent and, at a higher dose, essentially reduced the per cent of degenerated embryos. In cultured embryos, IGF-I at both doses elevated the cell number compared with non-cultured embryos. However, in comparison with embryos cultured without IGF-I, only the higher IGF-I dose resulted in elevating the embryo cell number. The TUNEL index was significantly lowered by IGF-I treatment. Thawed embryos were mostly of the grade III actin type and fewer (12%) had grade II actin, whilst no grade I actin embryos were noted. The addition of IGF-I resulted in the appearance of grade I actin embryos (8.33 and 6.9% for 10 and 100 ng/ml, respectively). These observations indicate that the addition of IGF-I during post-thaw culture can improve the quality of bovine cryopreserved embryos.

Keywords: Cattle, Embryo, IGF-I, Post-thaw quality

Introduction

Cryopreservation of cattle embryos has been widely used in reproductive research and in commercial breeding programmes. Cryopreservation procedures still cause significant morphological and biochemical alterations, which may lead to cell death and loss of embryo viability. Loss of viability can be attributed to the type and concentration of cryoprotectant, the freezing protocol, species, genotype, development stage of the embryo (Fabian *et al.*, 2005) and the length of storage in liquid nitrogen (Marquez-Alvarado *et al.*, 2004).

Reliable information about embryo viability can be provided either by ultrastructural analysis by electron microscopy or by embryo transfer to recipient females, but these methods may not be available practically in every laboratory. Cryotolerance may be a useful indicator of blastocyst quality (Rizos *et al.*, 2001). As a functional criterion for the evaluation of embryo viability after cryopreservation, a post-thaw cleavage up to blastocyst stage (Popelkova *et al.*, 2005; Makarevich *et al.* 2008), embryo cell number and embryo diameter (Popelkova *et al.*, 2009), proliferation (PCNA) index (Markkula *et al.*, 2001) or number of apoptotic (TUNEL) cells (Marquez-Alvarado *et al.*, 2004; Makarevich *et al.*, 2008) and the state of actin cytoskeleton (Tharasanit *et al.* 2005; Makarevich *et al.*, 2008) have been used. The diameter of the embryo has

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been proposed as an indicator of the viability of bovine expanded blastocysts (Mori *et al.*, 2002). All above cited studies reported results obtained on *in vitro* produced embryos, but their viability potential and morphology may differ from those in *in vivo* recovered embryos (Wright & Ellington, 1995; Greve *et al.*, 1993).

One potential way to improve post-thaw survival and post-transfer efficiency is to modify post-thaw culture conditions to more closely simulate embryo physiology *in vivo.* It has become evident that macromolecules are an essential component of the embryo culture media. One macromolecule that can regulate embryonic development may be insulin-like growth factor I (IGF-I), which has been reported to have beneficial effects on bovine embryo development (Block, 2007). There are a number of studies that indicate that IGF-I improves bovine embryo production *in vitro* after its supplementation at the concentrations of 10–200 ng/ml (see review, Velazquez *et al.*, 2009). In particular, IGF-I promoted development to the blastocyst stage, increased blastocyst cell number and decreased the proportion of apoptotic blastomeres in *in vitro* produced embryos (Byrne *et al.*, 2002; Makarevich & Markkula, 2002; Block, 2007). On the other hand, some authors reported no beneficial effects of IGF-I treatment on *in vitro* produced fresh (Quetglas *et al.*, 2001; Block *et al.*, 2008) or frozen–thawed (Hernandez-Fonseca *et al.*, 2002) bovine embryos. These contrasting results may be explained by differences in culture media, protein supplementation, the IGF-I concentration or other factors. However, effect of IGF-I supplemented during post-thaw culture on quality of *in vivo* recovered cattle embryos has not been reported previously.

The goal of this study was to examine the effect of two concentrations of IGF-I added during post-thaw culture of cryopreserved bovine *in vivo* recovered embryos on their preimplantation viability and quality. We determined advance in development of embryos up to the blastocyst stage, embryo cell number, apoptosis occurrence and quality of actin cytoskeleton.

Materials and methods

In vivo recovery of cattle embryos

Dairy cows (Czech Fleckvieh cattle breed) were superovulated with the preparation of pFSH (Folicotropin (Spofa, Prague, Czech Republic) at dose of 500 I.U.) and $PGF_{2\alpha}$ (Oestrophan (Lečiva, Prague, Czech Republic) at dose of 0.5 mg). The embryos at the morula or compact morula stage were recovered non-surgically by uterine flushing with DPBS (Dulbecco's phosphate-buffered saline) supplemented with 2% of fetal calf serum on the fifth to sixth days following insemination. Embryos were evaluated under a stereomicroscope according to IETS Manual (1990) and only embryos characterized by good morphology (grades 1 and 2) were selected for cryopreservation.

Freezing–thawing of embryos

The embryos were prepared for cryopreservation by 10–15 min equilibration in an H-MEMD medium (Sevapharma, Prague, Czech Republic) with 10% v/v glycerol added. Thereafter, the straws filled with embryos were immediately placed into the chamber of a programmable freezer and were frozen to −33 °C according to Řiha *et al.* (2002). Then the straws were placed into liquid nitrogen (LN_2). The straws with embryos had been stored in LN_2 from 1989–1990 until 2008.

Thawing of the embryos was done by plunging the straws into the waterbath (at 35 °C) for 1 min. Glycerol was removed from the embryos by a three-step washing procedure: first step: in 6% glycerol and 0.3M sucrose; second step: in 3% glycerol and 0.3M sucrose; third step: in 0.3M sucrose without glycerol (all steps in an H-MEMD medium, 5 min for each step). Then the embryos were washed three times in fresh H-MEMD medium. The embryos were divided into four groups: the group 1: without post-thaw culture (control; 0 h); group 2: with post-thaw culture for 48 h (control for IGF-I); the groups 3 and 4¨cultured for 48 h in the presence of human recombinant IGF-I (Sigma) at concentrations of 10 ng/ml or 100 ng/ml, respectively. The embryos from groups 2, 3 and 4 were transferred into 35-µl droplets of SOF (synthetic oviduct fluid) culture medium (Minitub) plus added sodium pyruvate, fetal calf serum (5%), gentamycin, essential amino acids, non-essential amino acids and cultured in a humidified atmosphere with 5% O_2 and 5% CO_2 at 39 °C for 48 h.

The embryos were examined under a stereomicroscope for post-thaw development after 24 h and 48 h of culture. Thereafter, the embryos were analyzed for the occurrence of apoptosis (TUNEL assay), embryo cell number and quality of actin cytoskeleton.

Analysis of embryos for TUNEL, embryo cell number and actin cytoskeleton

The embryos were washed three times for 5 min in PBS–PVP washing solution (PBS with 4 mg/ml polyvinylpyrrolidone; Sigma-Aldrich). Then the embryos were fixed in 3.7% neutrally buffered formalin (Fluka) for 5 min and in 70% ethanol for 10 min. Permeabilization was done by a 15-min incubation of embryos in 0.5% Triton X-100 in PBS. The embryos were processed for TUNEL using the MEBSTAIN Direct Apoptosis Detection Kit (IM3171, Immunotech) according to the product manual. Briefly, fixed and permeabilized embryos were incubated at 37 °C in TdT-labelling mixture (TdT buffer, FITC–dUTP and

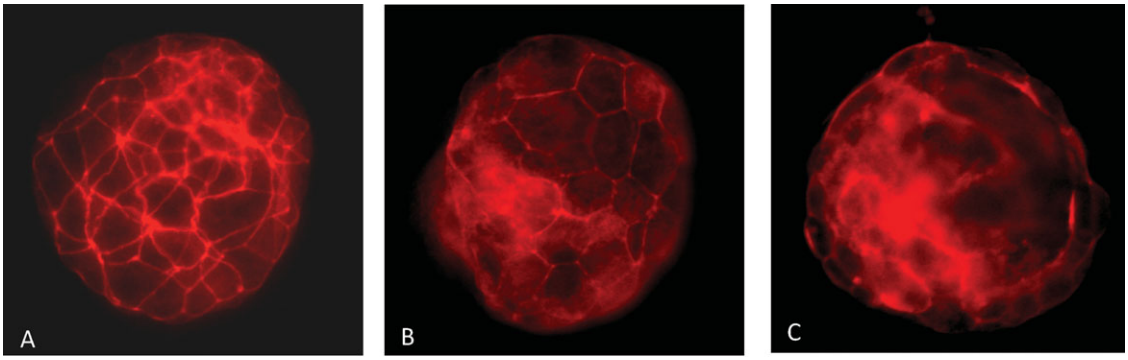

Figure 1 Cattle embryos stained for actin cytoskeleton. (*A*) Grade I: sharply stained actin filaments of reticular shape in cell borders. (*B*) Grade II: blastomeres with less pronounced actin filaments without sharp borders on membranes. (*C*) Grade III: large areas lacking actin staining or visible actin is largely aggregated in intra-cytoplasmic clumps. (See online for a colour version of this figure.)

TdT) for 1 h. Following this incubation the TUNEL reaction was stopped by washing embryos three times in a PBS–PVP solution and then the embryos were put into phalloidine–TRITC conjugate (Chemicon International), for labelling actin filaments, for 45 min. Thereafter the embryos were transferred onto coverslips and covered with 5 µl of Vectashield antifade mounting medium, containing DAPI stain (Vector Laboratories). The coverslip was attached to a microslide using nail polish. All treatments were performed at ambient temperature. The samples were stored at −20 °C until fluorescence analysis.

TUNEL indexes were determined on the basis of proportion of TUNEL-positive nuclei (green fluorescence) to total embryo cell number (DAPI-stained nuclei), which have been counted under the Leica fluorescence microscope (Leica Microsystems, Germany) using specific wavelength filters.

The actin cytoskeleton was classified as belonging to three grades according to Tharasanit *et al.* (2005) on the basis of appearance of actin filaments in horse embryos, with our adaptation to bovine embryos. Grade I (best quality): sharply stained actin filaments of reticular shape in cell borders (Fig. 1*A*); grade II (fair quality): blastomeres with less pronounced actin filaments without sharp borders on membranes (Fig. 1*B*); grade III (poor quality): large areas lacking actin staining or visible actin largely aggregated into intracytoplasmic clumps (Fig. 1*C*).

Statistics

The experiment was performed five times. The influence of post-thaw culture and IGF-I treatment on the development of the embryos was analyzed by chi-squared test. A one-way ANOVA and Tukey-test were used to analyze differences in TUNEL index between groups. As the variable 'TUNEL index' was normally distributed, a log-transformation of original values was not done. The hypothesis of normality was rejected for 'TUNEL cells per embryo' and for 'embryo cell number'. For these two variables the log-transformation of original values was used. The treatment effect on actin cytoskeleton quality was tested using the Mann–Whitney *U*-test. All calculations were performed using the SAS software package (SAS Institute, 2002).

Results

The embryos used in this study were frozen within the period of 1989–1990. In total, 258 embryos at the morula or compact morula stages were thawed, of these 221 embryos were selected for the experiment and the rest were rejected because of damage to the zona pellucida or embryo destruction during cryopreservation. Most embryos ($n = 171$) were post-thaw cultured for 48 h and 50 embryos were fixed immediately after thawing for fluorescence analysis.

Following post-thaw culture, about 41% of thawed embryos developed up to advanced blastocyst stage (blastocyst plus expanded blastocysts). This per cent was significantly increased when IGF-I (10 ng/ml or 100 ng/ml) was added to the culture medium. IGF-I at the higher dose (100 ng/ml) significantly decreased the percentage of degenerated/cleavage-arrested embryos from 35.3% in the control to 17.2% (Table 1.).

Average embryo cell number in the embryos immediately after thawing was about 67. In post-thaw cultured embryos, IGF-I, given at both concentrations, significantly elevated the cell number compared with non-cultured embryos (Table 2). However, when compared with embryos cultured for 48 h without

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Table 1 Effect of IGF-I on development of bovine embryos following 48 h post-thaw culture

Groups	Total no. embryos, *n*	Of them: morula, *n*/%	Blastocysts, *n*/%	Expanded blastocysts, *n*/%	Total no. blastocysts, *n*/%	Degenerated, *n*/%
IGF-I 0 ng/ml (control)	51	12/23.53	12/23.53	9/17.65	21/41.18	18/35.29
IGF-I 10 ng/ml[a]	62	0/0	3/4.84	39/62.9	42/67.74	20/32.26
IGF-I 100 ng/ml[a]	58	0/0	20/34.48	28/48.28	48/82.76	10/17.24

[a]Significant differences at $p < 0.001$ compared with control (chi-squared test).

Table 2 Effect of 48 h post-thaw culture and IGF-I on the cell number and apoptosis occurrence in bovine embryos, (mean ± SEM)

Groups	No. embryos	Embryo quality parameters		
		Embryo cell number, $n^{a–d}$	TUNEL cells/embryo, $n^{a–d}$	TUNEL index $\%^{a–d}$
0 h post-thaw culture	50	$66.8 \pm 7.96^{a,b}$	$12.4 \pm 2.46^{a,b}$	22.22 ± 0.59^{a}
48 h post-thaw culture				
IGF-I 0 ng/ml	50	$92.5 \pm 12.76^{a,c}$	$14.40 \pm 2.94^{a,c}$	17.7 ± 1.59^{b}
IGF-I 10 ng/ml	60	$105.8 \pm 15.03^{c,d}$	$10.2 \pm 3.1^{a,c}$	7.56 ± 1.91^{c}
IGF-I 100 ng/ml	58	112.6 ± 3.25^{d}	8.4 ± 0.93^{c}	$6.92 \pm 1.03^{c,d}$

[a–d]Values in a column with different superscript letters differ significantly (one-way ANOVA and Tukey test, $p < 0.05$).

Table 3 Distribution of bovine embryos to quality grades of actin cytoskeleton, (n/%)

Groups	No. embryos	Actin grades		
		I	II	III
0 h post-thaw culture[a]	50	0	6/12.00	44/88.00
48 h post-thaw culture				
IGF-I 0 ng/ml[b]	50	0	28/56.0	22/44.00
IGF-I 10 ng/ml[c]	60	5/8.33	35/58.33	20/33.33
IGF-I 100 ng/ml[c]	58	4/6.90	36/62.07	18/31.03

[a–c]Differences between the groups are significant at $p < 0.05$ (Mann–Whitney *U*-test).

IGF-I, only the higher IGF-I dose (100 ng/ml) was effective in elevating the embryo cell number. The number of TUNEL-positive cells per embryo was not significantly changed by the addition of IGF-I compared with the IGF-I-free embryo group. The TUNEL index of thawed embryos was significantly decreased following 48 h culture (17.7%), when compared with the non-cultured control (22.2%). IGF-I, given at both tested doses, significantly lowered the apoptotic (TUNEL) index compared with the IGF-I-free group.

Frozen–thawed embryos mostly exhibited grade III actin quality (88%) and fewer (12%) were in the grade II actin group (Table 3.). No grade I actin embryos were noted after thawing. Following 48 h-post-thaw culture most embryos (56%) belonged to the grade II actin group and the rest (44%) to the grade III actin group. Therefore the addition of IGF-I at both tested concentrations changed the distribution of actin grades compared with the group without IGF-I: grade I actin embryos appeared in both IGF-I supplemented groups. No significant differences in the actin grade distribution have been determined between both IGF-I concentrations.

Discussion

The viability of animal embryos after cryopreservation can affect efficiency of embryo transfer and birth rate. Therefore, many efforts have been undertaken for several decades to improve post-thaw quality and viability of mammalian embryos. One of approaches is to add various substances with growth-stimulating and/or membrane-stabilizing effects either before freezing or post-thawing or during both periods. IGF-I is a growth factor that is known to stimulate embryo cell proliferation and to suppress apoptosis (Byrne *et al.*, 2002; Makarevich & Markkula, 2002). However, an effect of IGF-I during post-thaw culture on quality of cattle embryos has not been reported.

Supplementation of post-thaw culture medium with IGF-I in our experiments was beneficial for the embryo survival by stimulating blastocyst yield and embryo cell number, while suppressing TUNEL-related apoptosis. Effects of IGF-I to suppress apoptosis in

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bovine IVP embryos have been reported previously (Byrne *et al.*, 1999, 2002; Makarevich & Markkula, 2002; Sirisathien & Brackett, 2003), but IGF-I effect on apoptosis in bovine frozen–thawed embryos has not been proved (Hernandez-Fonseca *et al.*, 2002). However, Block *et al.* (2008) reported that increased survival of embryos treated with IGF-1 does not appear to be due to effects on cell number or apoptosis but due to the altering expression of several transcripts that may be important for embryo development and survival following transfer.

In our study, IGF-I, given at both concentrations, promoted embryo development up to advanced blastocyst stage. Generally, increased blastocyst yield may not be correlated with higher developmental potential. Therefore, other criteria for the evaluation of embryo viability after cryopreservation are required. In our study we used invasive parameters of embryo quality, such as embryo cell number, actin cytoskeleton and apoptosis occurrence (TUNEL index). However, such a system of quality evaluation has a caveat that analyzed embryos are no longer alive and they cannot be examined for post-transfer developmental potential.

In our previous study on fresh bovine *in vitro* produced embryos the apoptotic (TUNEL) index was about 5% (Makarevich & Markkula, 2002). The TUNEL index in the frozen–thawed embryos in the present study was in the range of 7–22%. One of the factors that can cause this difference may be a cryopreservation process.

Apart from the damage caused during cryopreservation, external factors (such as a decrease in the amount of liquid nitrogen to levels below recommended during storage, excessive manipulation with the racks where embryos are placed, and transport and handling of embryos) might result in greater losses, causing irreversible damage to embryo cells (Marquez-Alvarado *et al.*, 2004). The latter mentioned authors used the TUNEL technique to analyze the viability of bovine frozen–thawed embryos and they showed that embryos with a shorter storage time (1–2 years) presented a lower number of TUNEL-positive cells compared with embryos stored for longer times (4–5 years). The embryos taken for the present study were frozen about 17–18 years ago. Probably this fact caused the lower quality of thawed embryos. We observed a high dead cell index (about 22%) and low cytoskeleton quality (grades II or III) in bovine embryos. However, following 48 h of culture most of frozen–thawed embryos were able to regenerate, proved by the increase in embryo cell number, decrease in dead cell (TUNEL) index and the change in half of the embryos (44%) from grade III to the grade II actin quality. In our study, we did not perform analysis on freshly flushed bovine embryos to compare these with frozen–thawed ones. However, Marquez-Alvarado *et al.* (2004) in their study showed a highly significant increase in the number of apoptotic cells in frozen embryos compared with fresh embryos, indicating that cryopreservation per se can cause damage that compromises embryo viability. As *in vivo* recovered cattle embryos were used both in the above mentioned study and in our present work, this hypothesis may be also applicable to our results.

The probable reason for reduced embryo viability following freezing and thawing is the disruption of the cytoskeleton, as a result of intracellular ice formation (Dobrinsky, 1996). Consequences of cryopreservation on actin cytoskeleton quality have been studied on equine (Tharasanit *et al.*, 2005) and rabbit (Makarevich *et al.*, 2008) frozen–thawed embryos. In the study by Tharasanit *et al.* (2005), no grade I actin embryos were observed following cryopreservation either immediately after thawing or after 6 h culture with cytochalasin B–stabilizator of the cytoskeleton. Similarly in our study, no grade I actin embryos were noted either immediately after thawing or after post-thaw culture. However, culture in the presence of IGF-I (at both concentrations) resulted in a small proportion (6.9 and 8.3%) of grade I actin embryos and most of embryos redistributed to grade II actin quality. This finding indicates some regenerating effect of IGF-I on the embryo cytoskeleton. Our results on actin quality in bovine frozen–thawed embryos are substantially different from those obtained in rabbit vitrified embryos (Makarevich *et al.*, 2008). In the latter study, 58% and 70% of vitrified–warmed embryos (depending on vitrification protocol, respectively) were classified as belonging to the best (grade I) actin quality. This difference may be explained by different lengths of embryo storage in liquid nitrogen and/or by different cryopreservation techniques (vitrification versus slow freezing) or animal species. Rabbit embryos, in contrary to bovine embryos, have been stored in liquid nitrogen for a substantially shorter time – about several days (Makarevich *et al.*, 2008). Cytoskeleton damage probably explains the freezing-induced decrease in viability of bovine frozen–thawed embryos, similarly to those previously reported for cryopreserved horse embryos (Tharasanit *et al.*, 2005).

Survival of embryos after freezing–thawing may be best characterized by the subsequent embryo transfer to synchronized recipient females. However, in the present study we did not perform post-thaw embryo transfer, as all thawed embryos were used for invasive evaluation (TUNEL, actin cytoskeleton) and, therefore, were no longer alive. Nevertheless, in earlier experiments that have been performed at the same laboratory (Řiha *et al.*, 2002), from 71 bovine embryos recovered *in vivo* from the same cattle breed, frozen by the same technique and stored in the same

Dewar's containers as in the present study, 66.2% pregnancies (47 pregnant recipients) were established following direct transfer without post-thaw culture. Therefore, there is an assumption, that in the case of post-thaw embryo culture in the presence of IGF-I, the pregnancy rate following transfer of such embryos could be increased over the abovementioned value.

Our observations lead to following conclusions: (1) evaluation of TUNEL index in conjunction with the actin cytoskeleton quality can provide additional information on post-thaw developmental potential of bovine embryos and possible effect of a treatment; and (2) IGF-I can improve quality of frozen–thawed bovine embryos when added during post-thaw culture.

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