Collection of oocytes from donors in the growth phase of follicular development can enhance the production of bovine embryos for cryopreservation

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ABSTRACT: The present study was designed to compare the efficiency of bovine embryo production for cryopreservation between oocytes collected from donors in the growth phase of follicular development (GPFD) and those recovered from donors in the undefined phase (UPFD). Cyclic cows, Czech Siemental or Holstein dairy breeds, 4–6 years of age, slaughtered at the local abbatoir were used. They were divided into two groups based on ovarian morphology: I. GPFD donors with ovaries corresponding to the growth phase of the first follicular wave (estrus cycle days 3–4; n = 52), and II. UPFD donors with ovaries in any other phase of follicular development (undefined estrus cycle days; n = 89). A total of 3 771 oocytes were collected and 1 134 embryos were prepared as two separate populations by standard protocol. In total 352 excellent or good quality embryos at the early, advanced or expanded blastocyst stage from both donor groups were pooled and used for cryotolerance assessment. They were frozen on day 7 (D7) or day 8 (D8) after fertilization by the standard procedure. After thawing, the embryos were cultured for 72 h to the hatching stage. The percentages of both D7 embryos and advanced blastocysts were significantly higher ($P \leq 0.01$) for oocytes collected from GPFD donors than for oocytes collected from UPFD donors (33.7 vs 28.6% and 43.0 vs 29.5%, respectively). The percentages of excellent or good quality embryos obtained from both D7 embryos and fertilized oocytes were significantly higher ($P \leq 0.01$) for oocytes collected from GPFD donors than for oocytes collected from UPFD donors (63.6 vs 49.4% and 21.4 vs 14.1%, respectively). The post-thaw survival rates were significantly higher ($P \leq 0.01$) for D7 than D8 embryos (80.4 vs 66.3%). In relation to the developmental stage, the development and hatching rates were significantly higher ($P \leq 0.01$) for D7 than D8 early blastocysts (75.0 vs 41.2% and 50.0 vs 5.9%, respectively) and for D7 than D8 advanced blastocysts, (73.7 vs 57.1 and 52.6 vs 28.6%, respectively). No differences were found between D7 and D8 expanded blastocysts after freezing-thawing. In conclusion, the collection of oocytes from donors in the growth phase positively influenced the *in vitro* production of bovine embryos for cryopreservation. The development of embryos produced with oocytes from GPFD donor group was accelerated and more excellent and good advanced blastocysts with greater cryotolerance were produced on day 7 after fertilization, as compared to embryos produced with oocytes from the UPFD donor group.

Keywords: follicular development; bovine oocyte; *in vitro* embryos; cryopreservation

The application of biotechnology in cattle breeding has allowed us to reduce the generation interval and to increase selection pressure in animal populations on the basis of more objective evaluation of the genotype. It facilitates more effective utilization of the reproductive potential of animals, makes a broader range of parental combinations available and thus allows for modification of the animal population in a desired way.

The prerequisite for achieving these goals involves efficient cryopreservation methods for long-term storage of embryos, exchange of genetic material and creation of genetic resources. Today the embryos obtained by superovulation can be fro-

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zen without any significant loss of viability; about 50% of them develop after transfer to synchronized recipients, which is comparable with the development of fresh embryos (Niemann, 1991; Riha et al., 2004). However, the utilization of genetically valuable parents can be made more effective with the use of cryopreserved embryos produced by *in vitro* fertilization of oocytes collected from living or slaughtered cows.

Although much progress has been made in bovine embryo cryopreservation, the development of frozen-thawed *in vitro* produced embryos is still considerably lower than that of embryos from superovulated donors (Hoshi, 2003). *In vitro* produced embryos show much less tolerance to low temperatures and therefore their cryopreservation is less successful (Leibo and Loskutoff, 1995; Vajta et al., 1997; Rizos et al., 2003). After thawing the embryos show a partial loss of microvilli, disruption of plasma membranes, as well as changes in mitochondria, the endoplasmic reticulum and zona pellucida. Nuclei, on the other hand, appear to be resistant to freezing (Ohboshi et al., 1998; Baguisi et al., 1998; Moreira da Silva and Metelo, 2005). However, the damage due to cryopreservation is to some extent reversible; less damaged embryos can regenerate and reorganize their blastocoel (Vajta et al., 1997). The lower resistance of *in vitro* produced embryos, as compared with embryos recovered *in vivo*, may be due to some differences in morphological and metabolic properties, including kinetics of embryonic development and early gene expression (Holm and Callesen 1998; Abe and Hoschi, 2003).

Attempts have been made to improve the production of high quality embryos for cryopreservation and their survival after thawing by the modification of conditions for oocyte maturation and cultivation of early embryos, as well as the selection of embryos for cryopreservation on the basis of their developmental kinetics (Massip et al., 1995). When the maturation of bovine oocytes was stimulated, the development of embryos accelerated and their morphological quality was improved, which resulted in a higher yield of embryos suitable for cryopreservation (de Matos et al., 1996; Mtango et al., 2003). Culture conditions have been shown to be a very important factor for survival of embryos after cryopreservation (Carnegie et al.,1999; Hochi et al., 1999; Otoi et al., 2000; Cho et al., 2002; Abe and Hoschi 2003; Galli et al., 2003; Rizos et al.,2003). It could be shown that, that it is possible to obtain more developed embryos of higher morphological quality that were more resistant to the cryopreservation procedure by modification of the culture condition.

The intrinsic quality of each oocyte is one of the important factors affecting development and morphology of the embryo (Lonergan et al., 1994, 2003; Sirard and Blondin, 1996; Rizos et al., 2002). There is a general agreement that the developmental competence of bovine oocytes after fertilization is related to the functional status of follicles from which the oocytes originated (Machatkova et al., 1996; Hagemann et al., 1999; de Wit et al., 2000; Hendriksen et al., 2000). During the estrous cycle, the follicle population changes under the influence of follicular waves. We have shown by our previous work that the development of *in vitro* produced embryos improves if oocytes from donors are collected in the growth phase of follicular waves (Machatkova et al., 2000, 2004).

The present study was designed to compare the production efficiency of embryos suitable for cryopreservation between oocytes collected in the growth phase of the first follicular wave and those from an undefined phase of follicular development.

MATERIAL AND METHODS

Donor groups and oocyte collection. Cyclic cows, Czech Siemental or Holstein dairy breeds, 4–6 years of age, slaughtered regardless of their estrus cycle stage at the local abbatoir, were used as donors. Prior to oocyte collection the ovaries of each donor were transported at 29°C to the laboratory and checked in terms of their morphology. According to it, the donors were divided into two groups: cows with ovaries corresponding to the growth phase of the first follicular wave (estrus cycle day 3 and 4) were included in the GPFD donors (n = 52) and cows with ovaries corresponding to any other phase of follicular development (undefined estrus cycle days) made up the UPFD donors (n = 89). Evaluation criteria for the GPFD donors were a hemorrhagic *corpus luteum* with signs of ovulation and a population of follicles with no follicle larger than 11 mm located on the ovaries. Oocyte-cumulus complexes were collected, by slicing the ovarian cortex. They were collected separately as two oocyte populations. All collected oocytes, except for markedly atretic or denuded oocytes, were used in the experiments.

Oocyte maturation, fertilization, and cultivation. Oocytes were matured in 500 µl of TCM 199 medium with the addition of 0.20mM sodium pyruvate, 50 IU/ml penicillin, 50 µg/ml streptomycin (Sigma Chemicals Co., Prague, Czech Republic), 5% ECS (estrus cow serum) and gonadotropins (P.G.600, 15 IU/ml; Intervet, Boxmeer, Holland) in culture dishes (Nunclon Intermed, Roskilde, Denmark) for 24 hours.

They were fertilized with spermatozoa isolated by the swim-up method from frozen-thawed semen of a standard bull, using SP-TALP medium. The same semen batch was used in the experiments. Fertilization was carried out in IVF-TALP medium containing 1×10^6/ml spermatozoa and 10 µg/ml heparin.

Cumulus cells were removed from oocytes by vortexing at 24 h after fertilization. Presumptive zygotes were transferred to a Buffalo rat liver cell line monolayer (BRL cell line, ATCC, Rockville, MD, USA) and cultured in B2 medium with 10% ECS. All cultures were incubated at 38.8°C in a humidified atmosphere of 5% CO_2 and air.

Evaluation of embryo development. The embryo development was expressed as the percentage of morula-blastocysts developed from fertilized oocytes on day 7 (D7). The kinetics of blastocyst formation was expressed as percentages of morula-to-early blastocysts or advanced-to-expanded blastocysts from D7 embryos. The suitability of embryos for cryopreservation was expressed as the percentage of excellent and good embryos from both D7 embryos and fertilized oocytes. They were classified by usually used morphologically criteria.

Embryo freezing and thawing. In total 352 excellent or good quality embryos at the early, advanced or expanded blastocyst stage from both donor groups were pooled and used for cryotolerance assessment. They were frozen on day 7 or day 8 after fertilization by the following procedure: The embryos were placed in 10% glycerol (v/v) in TCM 199 medium with 10% ECS and equilibrated for 5 min at room temperature. Subsequently, they were transferred into a column of freezing medium in 0.25-ml straws and allowed to stand for 10–15 min at room temperature. The straws were placed in a programmable freezer at –7°C and, after 10 min, were seeded. After another 10 min they were cooled to –35°C at a rate of 0.3°C/min; then they were stored in liquid nitrogen until use.

Frozen straw were thawed by holding it for 10 s in the air and then placing it in a 30°C water bath. Glycerol was washed out by the three-step method at 5-minute intervals at room temperature. The thawed embryos were transferred first to 6.6% glycerol (v/v) and 0.3M sucrose, then to 3.3% glycerol and 0.3M sucrose and finally to 0.25M sucrose in MEM (Earle' salt) medium. Subsequently, the embryos were washed with culture medium and transferred onto a BRL cell line monolayer in B2 medium with 10% ECS. They were evaluated at 24-hour intervals for 72 h, and the rate of development was expressed in percentages of embryos developed and hatched from the frozen-thawed embryos.

Statistical analysis. The data was analyzed by the Chi-square test, using the SPSS, Version 6.1 for Windows software (SPSS, Inc) and the ANOVA procedure.

RESULTS

Embryo development

Differences in both the embryo development rate and blastocyst formation were found between the oocytes recovered from the GPFD donors and those from the UPFD donors. The development rate of D7 embryos from oocytes of GPFD donors was significantly higher ($P \leq 0.01$) than the rate of D7 embryos from UPFD donors. Blastocyst formation was accelerated in the oocytes from the GPFD do-

Table 1. Development and blastocyst formation of D7 embryos derived from oocytes collected from donors in the growth or the undefined phase of follicular development

Follicular development phase	Oocytes fertilized (*n*)	Embryos developed		Developmental stage			
				morula-to-early blastocyst		advanced-to-expanded blastocyst	
		n	%	*n*	%	*n*	%
Growth	1 083	365	33.7[a]	208	57.0[a]	157	43.0[a]
Undefined	2 688	769	28.6[c]	542	70.5[c]	227	29.5[c]

[a,c]values with different superscripts in the same column differ significantly ($P \leq 0.01$)

Table 2. Rates of excellent and good D7 embryos derived from oocytes collected from donors in the growth or the undefined phase of follicular development

Follicular development phase	Excellent and good embryos/developed embryos		Excellent and good embryos/fertilized oocytes	
	n	%	*n*	%
Growth	232/365	63.6[a]	232/1 083	21.4[a]
Undefined	380/769	49.4[c]	380/2 688	14.1[c]

[a,c]values with different superscripts in the same column differ significantly ($P \leq 0.01$)

nors in comparison with those from the UPFD donors. At 7 days after fertilization, the percentage of advanced-to-expanded blastocysts was significantly higher ($P \leq 0.01$) and the percentage of morula-to-early blastocysts was significantly lower ($P \leq 0.01$) for the oocytes collected from the GPFD donors than for those from the UPFD donors (Table 1).

Embryo morphology

On evaluation of morphological characteristics of D7 embryos developed from oocytes of the two donor groups, differences were found in that the morphology was improved for the GPFD donors. The rates of excellent and good D7 embryos from both the developed embryos and the fertilized oocytes were significantly higher ($P \leq 0.01$) for the GPFD than the UPFD donors (Table 2).

Embryo survival after freezing

To verify the viability of *in vitro* produced embryos after cryopreservation, the embryos of both groups were pooled, frozen, thawed and cultured for 72 h to the hatching stage. The survival rate after thawing was significantly higher ($P \leq 0.01$) in D7 than in D8 embryos, as shown by the development and hatching rates presented in Table 3. In the frozen-thawed D7 embryos, both the development and hatching rates were similar for early, advanced and expanded blastocysts, but in the frozen-thawed D8 embryos the development and hatching rates were lower for early than for advanced blastocysts, as well as they were lower for advanced than for expanded blastocysts. The comparison of development and hatching rates between D7 and D8 embryos, as related to blastocyst stages, showed significant differences ($P \leq 0.01$) in the early and the advanced stage, but not in the expanded blastocyst stage (Table 4).

Table 3. Development and hatching rates for D7 and D8 frozen-thawed embryos

Cultivation of embryos (days)	Embryos frozen (*n*)	Development rate after freezing-thawing (%)		
		24 h	48 h	hatching
7	153	80.4[a]	79.1[a]	51.6[a]
8	199	66.3[c]	59.3[c]	36.2[c]

[a,c]values in the same column with different superscripts differ significantly ($P \leq 0.01$)

Table 4. Development and hatching rates for D7 and D8 embryos frozen-thawed at three blastocyst stages

Cultivation of embryos (days)	Embryos frozen (*n*)	Blastocysts		
		early	advanced	expanded
7	153	75.0[a] (50.0)[a]	73.7[a] (52.6)[a]	77.3[a] (63.6)[a]
8	199	41.2[c] (5.9)[c]	57.1[a] (28.6)[c]	73.1[a] (59.6)[a]

[a,c]values with different superscripts in the same column differ significantly ($P \leq 0.01$)

DISCUSSION

In the development of methods for cryopreservation of bovine *in vitro* produced embryos, attention has been focused, in the first place, on the investigation of the effects of various freezing procedures and cryoprotectants on the survival and further development of embryos after thawing. These studies have generally been based on conventional cryoprotective approaches involving slow-freezing of embryos, their appropriate thawing and a three-step washing of the cryoprotectants (Fahning and Garcia, 1992). In addition to these standard cryo-methods, some other cryoprotective techniques have been evolved. These are, for instance, the one-step method for direct embryo transfer, without washing out the cryoprotectant, and embryo selection after thawing or the technique of embryo vitrification based on extremely rapid embryo freezing directly in liquid nitrogen (Ishimori et al., 1993; Agca et al., 1998; Nedambale et al., 2004).

Even though these modified procedures have provided some technological advantages, *in vitro* produced embryos generally show higher viability when frozen by standard methods, as demonstrated by a lower occurrence of morphological changes after thawing (Moreira da Silva and Metelo, 2005). Also attempts at increasing the efficiency of cryopreservation by modifying classical freezing procedures have not brought about any reproducible results for *in vitro* produced embryos. Enhancement of *in vitro* embryo production by modification of culture media had not always had a positive effect on embryo cryotolerance (Imai et al., 2002).

The authors who studied factors involved in post-thaw survival of *in vitro* embryos demonstrated that the cryotolerance of an embryo was dependent on its morphology and the developmental stage at the moment of freezing (Fahning and Garcia, 1992; Balasubramanian et al., 1998).

Our results are in agreement with the conclusions of Massip et al. (1995) that, in addition to the morphology and the developmental stage of embryos, the kinetics of embryo development before freezing plays an important role in embryo survival after thawing. However, in contrast to these authors who recommended selection based on developmental kinetics for embryos before cryopreservation, we employed the selection of oocytes used for fertilization and embryo production. Our results agreed with those of Mahmoudzadeh et al., (1995), Vajta et al., (1995) and Dinnyes et al.(1999) that, in embryos of equal quality in terms of morphology, D7 embryos showed significantly higher cryotolerance than D8 embryos. We found that the highest difference in cryotolerance, more than 30 %, was between D7 and D8 embryos at the early blastocyst stage; this difference dropped to 20 % when the frozen-thawed embryos were at the advanced blastocyst stage and, at the expanded blastocyst stage, it did not exist. This implies that the most suitable for cryopreservation are fast developing embryos that, by day 7 have started to differentiate and have achieved at least the early blastocyst stage. On the other hand, D8 slowly developing early and advanced blastocysts seem to be more suitable for direct embryotransfer, because cryopreservation results in loss of their viability.

One way of achieving fast developing and high quality embryos is to improve the cytoplasmic maturation of oocytes (de Matos et al., 1996; Mtango et al., 2003). The supplementation of maturation medium with currently used additives need not necessarily give better results. If oocytes from a high performance donor are involved, they are recovered from different phases of follicular development and therefore present a very heterogeneous population in terms of meiotic and developmental competence that is a factor decisive for the kinetics and morphology of a developing embryo.

Our results suggest that the embryo development can be stimulated and the production of D7 embryos can be enhanced if they are derived from oocytes with a greater meiotic competence; these can be recovered from donors in the late stage of growth phase of follicular wave. At this stage on the ovaries, the number of follicles larger than 5 mm containing oocytes with a greater meiotic competence is increasing combined with an increase of the meiotic competence of oocytes from follicles smaller than 5 mm; consequently, the oocytes isolated at this time provide a homogenous population with improved developmental competence (Machatkova et al., 2004). The use of these oocytes increases embryo yields and improves the morphological quality of embryos and accelerates their development. First of all, this is important for the production of cryopreserved embryos from genetically valuable donors. The monitoring of follicular status of these donors by sonography, control of spontaneous or prostaglandin-synchronized heat and timing of oocyte collection can enhance the probability of acquiring of embryos suitable for cryopreservation.

In conclusion, the recovery of oocytes with greater meiotic competence in the growth phase of follicular development resulted in a faster embryo development and a higher yield of good quality embryos suitable for cryopreservation. The majority of embryos derived from these oocytes could be effectively frozen as the early blastocysts on day 7 after fertilization. This approach can become an alternative for the production of cryopreserved *in vitro* embryos from high performance donors.

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