In vitro production of embryos from high performance cows and the development of frozen-thawed embryos after transfer: a field study

M. Machatkova[1], P. Hulinska[1], Z. Reckova[1], K. Hanzalova[1], J. Spanihelova[2], R. Pospisil[3]

[1]Veterinary Research Institute, Brno, Czech Republic
[2]University of Veterinary and Pharmaceutical Sciences, Brno, Czech Republic
[3]Palacky University, Olomouc, Czech Republic

ABSTRACT: In this field study, embryos were derived from genetically highly valuable cows excluded from breeding due to reproductive disorders. Cows, 5 to 10 years old, of Czech Siemmental, Holstein Dairy and Beef Cattle breeds were used as oocyte donors. Oocytes were obtained either in the growth phase of the first follicular wave from cows with synchronized oestrus or in any other phases of follicular development from cows without oestrus synchronization. The embryos were prepared by a standard protocol described previously. The mean number of usable oocytes, transferable and freezable embryos per donor, and the mean percentage of usable, transferable and freezable embryos were assessed. The results were analyzed by Student's-*t* and Chi-squared tests. The embryos were frozen according to a slow freezing protocol. After thawing, they were transferred to recipients on Day 7 after oestrus. Irrespective of the breed, the mean numbers of usable oocytes and transferable and freezable embryos collected per donor were significantly higher ($P < 0.01$) for the synchronized than for the nonsynchronized donors (20.4 vs 11.7, 4.3 vs 1.0 and 3.2 vs 0.8, respectively). Similarly, the mean percentages of usable oocytes, transferable and freezable embryos were significantly higher ($P < 0.01$) for the synchronized than for the nonsynchronized donors (28.5% vs 20.5%, 20.9% vs 9.0% and 15.8% vs 6.5%, respectively). On comparison of the synchronized and nonsynchronized donors of each breed, the difference in the mean percentage of usable oocytes was significant ($P < 0.01$) in cows of all three breeds, the difference in the mean percentage of transferable embryos was significant in Czech Siemmental and Holstein Dairy cows ($P < 0.01$) and the difference in the mean percentage of freezable embryos was significant only in Holstein Dairy cows ($P < 0.01$). After the transfer of 41 frozen-thawed embryos and 43 fresh embryos, 20 heifers and 24 heifers became pregnant, respectively. In conclusion: (a) higher number of oocytes from infertile, genetically valuable cows was recovered in the growth phase compared with the other phases of follicular development; (b) greater development of these oocytes resulted in more embryos for transfer and cryopreservation; (c) the transfer of frozen-thawed and fresh embryos resulted in pregnancy rates of 48.8% and 55.8% , respectively.

Keywords: cattle; *in vitro* embryos; cryopreservation; embryo transfer

At present, reproductive biotechnologies are widely used for farm animal breeding. In cattle methods such as artificial insemination or superovulation and embryo transfer are generally employed, because they allow us to utilize the genetic potential of male and female animals more effectively. The *in vitro* production of embryos from oocytes of genetically highly valuable donors eliminated from breeding because of reproductive disorders can accelerate genetic progress. However, this requires an increase in the efficiency of usable oocyte collection, transferable embryo production and embryo

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survival after cryopreservation. Up to now, the production of embryos from oocytes of selected cows has not been very effective in comparison with the production of embryos from oocytes of populations of ordinary slaughter cows. Furthermore, *in vitro* produced embryos have lower cryotolerance than *in vivo* produced embryos (Enright et al., 2000; Dobrinsky, 2002), because of their higher sensitivity to low temperatures (Leibo and Loskutoff, 1993; Holm and Callesen, 1998). Embryo survival after cryopreservation has been shown to depend on the quality, age and developmental stage of an embryo at freezing (Fahning and Garcia, 1992; Mahmoudzadeh et al., 1995; Dinnyes et al., 1999; Ravindranatha and Reddy, 2001; Machatkova et al., 2006). Both the embryo quality and the kinetics of embryo development are related to the oocyte and, most importantly, to its meiotic and developmental competence (Rizos et al., 2002; Sirard et al., 2006; Lonergan, 2007). It has been documented that follicles present on the ovaries at the end of the growth phase of follicular wave can provide oocytes with greater meiotic and developmental competence (Hagemann et al., 1999; de Wit et al., 2000; Machatkova et al., 2004).

The efficiency of oocyte collection as well as of fresh and frozen embryo production were compared between the oocytes recovered in the growth phase and those recovered in the other phases of follicular development from genetically highly valuable cows excluded from breeding due to reproductive disorders. The frozen-thawed viability of these embryos was verified by the pregnancy rate in recipients after embryo transfer.

MATERIAL AND METHODS

Cows

A total of 52 cows, between 5 and 10 years of age, of Czech Siemmental (n = 15), Holstein Dairy (n = 29) and Beef Cattle (n = 8) breeds, were used as oocyte donors. In the first group of donors (n = 31), the oestrous cycle was synchronized by two doses of $PGF_{2\alpha}$ at an interval of 11 days and one dose of hCG (Pregnyl, 1 500 IU, Organon, Oss, The Netherlands). The oocytes were collected on Days 3–4 after oestrus in the growth phase of the first follicular wave. Subsequently, the cows were slaughtered. Evaluation criteria for the growth phase were the presence of a hemorrhagic *corpus luteum* with signs of ovulation and no dominant follicle on the ovaries larger than 11 mm in diameter. In the second group of donors (n = 21), the oestrus was not synchronized before slaughter and oocytes were collected in any of the other phases of follicular development.

Bulls

The frozen-thawed sperm of 21 elite bulls of Czech Siemmental (n = 6), Holstein Dairy (n = 8) and Beef Cattle (n = 7) breeds were used for oocyte fertilization.

Embryo production

Oocyte-cumulus complexes were isolated by total slicing of the ovarian cortex. Only good quality oocytes with homogenous dark cytoplasm and at least two layers of cumulus cells were considered usable. Embryos were prepared according to a standard protocol described previously (Machatkova et al., 2004). Oocytes were matured in TCM-199 medium (Earle's salt), supplemented with antibiotics, 0.20mM sodium pyruvate (Sigma-Aldrich, St. Louis, MO, USA), gonadotropins (P.G. 600, 15 IU/ml, Intervet, Boxmeer, The Netherlands) and 5% oestrus cow serum (ECS) for 24 hours.

Motile spermatozoa were isolated by the swim-up method from frozen-thawed sperm using modified Tyrode's medium (SP-TALP). They were coincubated with the oocytes at a concentration of 1×10^6 spermatozoa per ml in modified Tyrode's medium (IVF-TALP) supplemented with 10 µg/ml heparin for 20 hours. Presumptive zygotes were removed from cumulus cells, transferred onto a BRL cell line monolayer (Buffalo rat liver cells, ATCC, Rockville, MD, USA) and cultivated in Menezo B2 medium with 10% ECS. All procedures were carried out at 39°C with atmospheric conditions of 5% CO_2. Embryos, at earliest at an early blastocyst stage on Day 7, or advanced blastocyst stage on Day 8 were regarded as transferable.

Embryo cryopreservation

Only excellent blastocysts of good quality were selected for cryopreservation from transferable embryos. They were placed in freezing medium,

consisting of 10% glycerol (v/v) in TCM-199 medium with 10% ECS and equilibrated for 5 min at room temperature. Subsequently, each embryo was loaded into 0.25 ml straw in a column of freezing medium and allowed to stand for another 10–15 min at room temperature. Straws were placed in the programmable freezer at –7°C and, after 10 min, were seeded. After another 10 min the embryos were cooled to –35°C at a rate of 0.3°C/min; they were then plunged into liquid nitrogen.

Embryo transfer

The embryos were thawed by holding the straws for 10 s in the air and then placing them in a 30°C water bath for 30 s. The cryoprotective was removed using a three-step procedure (6.6% glycerol [v/v] and 0.3M sucrose; 3.3% glycerol and 0.3M sucrose; 0.25M sucrose in culture medium, TCM-199 with 10% ECS). Subsequently, the embryos were washed with culture medium and transferred as soon as possible to heifers on Day 7 after their oestrus. The pregnancy rate was assessed by palpation per rectum on Day 90 after transfer.

Statistical analysis

The mean number of usable oocytes, transferable and freezable embryos per donor, and the mean percentage of usable oocytes from collected oocytes, transferable and freezable embryos from usable oocytes were assessed. The results were analyzed by Student's-*t* and Chi-squared tests, using ANOVA SPSS Version 11.5 software for Windows (SPSS, Inc., Chicago, IL, USA).

RESULTS

Oocytes

The efficiency of oocyte collection from synchronized and nonsynchronized donors of different breeds is shown in Table 1. Regardless of the breed, the mean number of usable oocytes collected per donor and the mean percentage of usable oocytes were significantly higher ($P < 0.01$) for the synchronized than for the nonsynchronized donors. An evaluation of each breed revealed that the mean number of usable oocytes per donor was higher for the synchronized donors of Czech Siemmental, Holstein Dairy and Beef Cattle breeds, but that only in Czech Siemmental cows was the increase significant ($P < 0.05$). The difference in the mean percentage of usable oocytes between the synchronized and nonsynchronized donors was significant in cows of all breeds ($P < 0.01$, $P < 0.05$ and $P < 0.05$, respectively).

Table 1. Collection of oocytes from donors of different breeds in the growth phase and other phases of follicular development

Breed	Synchronized donors						Nonsynchronized donors					
	donors (*n*)	oocytes					donors (*n*)	oocytes				
		isolated		usable		usable/isolated (mean %)		isolated		usable		usable/isolated (mean %)
		total (*n*)	per donor (*n*)	total (*n*)	per donor (*n*)			total (*n*)	per donor (*n*)	total (*n*)	per donor (*n*)	
Czech Siemmental	9	592	65.8	182	20.2^{a1}	30.7^{a1}	6	366	61.0	64	10.7^{b1}	17.5^{c1}
Holstein Dairy	17	1 380	81.2	352	20.7^{a1}	25.5^{a2}	12	738	61.5	156	13.0^{a1}	21.1^{b1}
Beef Cattle	5	245	49.0	98	19.6^{a1}	40.0^{a3}	3	93	31.0	25	8.3^{a1}	26.9^{b2}
Total	31	2 217	71.5	632	20.4^{a}	28.5^{a}	21	1 197	57.0	245	11.7^{c}	20.5^{c}

Values in the same row with different superscripts are significantly different ($^{a–b}P < 0.05$; $^{a–c}P < 0.01$)
Values in the same column with different superscripts are significantly different ($^{1–2}P < 0.05$; $^{1–3,\ 2–3}P < 0.01$)

Table 2. Collection of transferable embryos from donors of different breeds in the growth phase and other phases of follicular development

Breed	Synchronized donors					Nonsynchronized donors				
	donors (*n*)	oocytes (*n*)	embryos			donors (*n*)	oocytes (*n*)	embryos		
			transferable		transferable/ oocytes (mean %)			transferable		transferable/ oocytes (mean %)
			total (*n*)	per donor (*n*)				total (*n*)	per donor (*n*)	
Czech Siemmental	9	182	43	4.8^{a1}	23.6^{a1}	6	64	8	1.3^{b1}	12.5^{c1}
Holstein Dairy	17	352	76	4.5^{a1}	21.6^{a1}	12	156	13	1.1^{a1}	8.3^{c1}
Beef Cattle	5	98	13	2.6^{a1}	13.3^{a2}	3	25	1	0.3^{a1}	4.0^{a1}
Total	31	632	132	4.3^{a}	20.9^{a}	21	245	22	1.0^{c}	9.0^{c}

Values in the same row with different superscripts are significantly different ($^{a-b}P < 0.05$; $^{a-c}P < 0.01$)
Values in the same column with different superscripts are significantly different ($^{1-2}P < 0.05$)

Transferable embryos

The efficiency of transferable embryo production from the synchronized and nonsynchronized donors of evaluated breeds is summarized in Table 2. Regardless of the breed, the mean number of transferable embryos produced per donor and the mean percentage of transferable embryos were significantly higher ($P < 0.01$) for the synchronized than for the nonsynchronized donors. The mean number of transferable embryos per donor was higher for the synchronized donors of all three breeds, but only in Czech Siemmental cows was the value significant ($P < 0.05$). The difference in the mean percentage of transferable embryos was significant in cows of Czech Siemmental and Holstein Dairy breeds ($P < 0.01$).

Freezable embryos

The efficiency of freezable embryo production from the synchronized and nonsynchronized donors of different breeds is presented in Table 3. Regardless of the breed, the mean number of freezable embryos produced per donor and the mean percentage of freezable embryos were significantly higher ($P < 0.01$) in the synchronized than the non-

Table 3. Collection of freezable embryos from donors of different breeds in the growth phase and other phases of follicular development

Breed	Synchronized donors					Nonsynchronized donors				
	donors (*n*)	oocytes (*n*)	embryos			donors (*n*)	oocytes (*n*)	embryos		
			freezable		freezable/oocytes mean %			freezable		freezable/ oocytes (mean %)
			total (*n*)	per donor (*n*)				total (*n*)	per donor (*n*)	
Czech Siemmental	9	182	31	3.4^{a1}	17.0^{a1}	6	64	5	0.8^{a1}	7.8^{a1}
Holstein Dairy	17	352	59	3.5^{a1}	16.8^{a1}	12	156	10	0.8^{a1}	6.4^{c1}
Beef Cattle	5	98	10	2.0^{a1}	10.2^{a1}	3	25	1	0.3^{a1}	4.0^{a1}
Total	31	632	100	3.2^{a}	15.8^{a}	21	245	16	0.8^{c}	6.5^{c}

Values in the same row with different superscripts are significantly different ($^{a-c}P < 0.01$)
Values in the same column with the same superscripts are not significantly different

synchronized donors. A separate evaluation of each breed showed that the mean number of freezable embryos per donor was higher for the synchronized donors of all three breeds, but that the difference was not significant. The difference in the mean percentage of freezable embryos was significant in Holstein Dairy cows ($P < 0.01$).

Embryo development after transfer

The frozen-thawed viability of the embryos was determined by their development after transfer into heifers on Day 7 after their oestrus. From the synchronized donors, 41 frozen-thawed embryos were transferred and 20 recipients became pregnant (48.8%). The transfer of 43 fresh embryos derived from the synchronized donors resulting in 24 pregnancies (55.8%) served as a control.

DISCUSSION

The application of new biotechnologies in cattle breeding can intensify the selective process and bring about a genetic benefit. The *in vitro* production of embryos from high performance donors, which cannot meet reproductive condition and must, therefore, be eliminated from breeding and slaughtered, is one of these methods. Combining this method with cryopreservation enhances the utilization of embryos from genetically valuable parents for both the preservation of genetic resources for the future and the utilization of embryo transfers within ongoing breeding programs. Up to now, however, the development of embryos produced *in vitro* from oocytes of selected donors and survival of these embryos after cryopreservation have been low compared with embryos produced *in vivo* from superovulated donors (Hasler, 2001; Dobrinsky, 2002).

In order to increase the developmental ability of *in vitro* produced and cryopreserved embryos, selection based on the kinetics of embryo development or the modification of conditions during maturation, cultivation and cryopreservation are recommended (Massip et al., 1995; Rizos et al., 2001, 2003; Cho et al., 2002; Imai et al., 2002; Mtango et al., 2003; Nedambale et al., 2004; Tominaga et al., 2007). New strategies based on metabolic manipulation with embryos before their cryopreservation have been described by Seidel (2006).

In our experiments, we made an effort to increase the production of embryos and to improve the survival of these embryos after freezing by the selection of oocytes with greater meiotic and developmental competence.

In our preliminary study involving slaughtered cows we found that oocytes recovered in the growth phase of the first follicular wave and fertilized by the spermatozoa of a standard bull produced more embryos with accelerated development and better morphological quality than oocytes recovered in the other stages of follicular development. We concluded that this approach could be used as an alternative method for the production of cryopreserved embryos from high performance cows (Machatkova et al., 2006).

In our field study with reproductively problematic donors, the 52 unfertile, genetically valuable cows provided a total of 877 usable oocytes, giving rise to 154 fresh embryos, 116 of which were used for cryopreservation (17.6% and 13.2%, respectively). Although the number of usable oocytes collected per donor was similar in all three breeds, the numbers of transferable and freezable embryos per donor obtained from Czech Siemmental and Holstein Dairy cows were twice the numbers of those obtained from Beef Cattle cows; this is because embryo development rates were significantly higher for the first two breeds than for the third one. For Czech Siemmental, Holstein Dairy and Beef Cattle cows, the mean proportion of transferable embryos derived from oocytes recovered in the growth phase of follicular development were 23.6%, 21.6% and 13.3%, respectively. These values are comparable with those reported by Galli et al. (2003) who obtained embryo development rates of 21.8% and 15.6% in oocytes collected from culled genetically valuable cows and heifers of one herd, respectively, and by Merton et al. (2007) who reached embryo development rates of 23.4 to 24.7% in oocytes collected by transvaginal aspiration from healthy pregnant heifers and first-parity Holstein Friesian cows.

It is generally known that the pregnancy rate after the transfer of cryopreserved embryos is lower than the rate after transfer of fresh embryos and that it is significantly lower after the transfer of frozen *in vitro* produced compared to the transfer of *in vivo* produced embryos (Hasler, 2001; Riha et al., 2002; Galli et al., 2003; Merton et al., 2007). Pregnancy rates of 28%, 30–40%, 32.8–39.2% and 35.6–40.8% were achieved by Dobrinsky (2002),

Hernandez-Fonseca et al. (2002), Hoshi (2003) and Merton et al. (2007), respectively, after the transfer of frozen-thawed embryos produced *in vitro* from slaughterhouse-collected and transvaginally aspirated oocytes.

In this study the pregnancy rates were 48.8% for cryopreserved and 55.8% for fresh embryos. A relatively low decrease in viability was found for frozen-thawed embryos compared to fresh embryos. The pregnancy rate was improved after the transfer of frozen-thawed embryos derived from oocytes with greater meiotic and developmental competence in comparison with the pregnancy rates after transfer of embryos derived from oocytes with heterogeneous meiotic and developmental competence, as used for embryo production by the authors mentioned above.

The results of this study confirm our assumption that the recovery of oocytes in a suitable phase of follicular development can be used to increase the production of fresh and frozen embryos from high performance donors and to improve the pregnancy rate after embryo transfer. In this case, however, the examination of follicular status of selected cows and the timing of oocyte collection for embryo preparation are important.

It can be concluded that (a) higher numbers of usable oocytes from infertile, genetically valuable cows can be recovered in the growth phase compared with any other phase of follicular development; (b) greater development of these oocytes results in more embryos for transfer and cryopreservation; (c) the pregnancy rate can be improved after the transfer of frozen-thawed embryos derived from oocytes with greater meiotic and developmental competence.

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Corresponding Author:

Ing. Marie Machatkova, CSc., Veterinary Research Institute, Hudcova 70, 621 00 Brno, Czech Republic
Tel. +420 533 331 418, fax +420 541 211 229, e-mail: machatkova@vri.cz