Vitrification of bovine oocytes: implications of follicular size and sire on the rates of embryonic development

Vahida M. Anchamparuthy · Arindam Dhali ·
Whitney M. Lott · Ronald E. Pearson ·
Francis C. Gwazdauskas

Received: 24 June 2009 / Accepted: 28 October 2009 / Published online: 11 November 2009
© Springer Science + Business Media, LLC 2009

Abstract

Purpose The objectives were to test how the source of oocytes and semen impacted vitrification of large numbers of bovine oocytes and subsequent IVF and early embryo development to test procedures that may assist with assisted reproductive technologies in humans.

Methods Bovine oocytes were vitrified from follicles of different diameters, small (≤ 4 mm) and medium (4 to 10 mm), using nylon mesh. Oocytes were exposed to the cryoprotectant composed of 40% (v/v) ethylene glycol, 18% (w/v) Ficoll-70, and 0.3 M sucrose in three stepwise dilutions. Thawing was conducted with a series of 0.5, 0.25 and 0.125 M sucrose dilutions in 20% fetal bovine serum. **Results** The cleavage (39.1% vs. 58.5%) and blastocyst rates (5.1% vs. 22.9%) were significantly lower for the vitrified oocytes. Follicle size had a significant impact on the development of embryos. Sires had significant effects on embryonic developmental rates.

Conclusions We conclude that differences in development exist due to follicle source and sire used for IVF after vitrification.

Keywords Bull · Embryo · Follicle · Oocyte · Vitrification

Introduction

Cryopreservation of oocytes is difficult in most animal species because of their large volume, high sensitivity to cooling, low surface area to volume ratio, high water content and low hydraulic conductivity [1]. Progress in the development of protocols for cryopreservation indicated that vitrification at an ultra rapid cooling rate is a promising approach for oocyte cryopreservation [2–4]. Problems currently associated with cryopreservation of bovine oocytes are the source of oocytes, stage of maturation, cumulus cell investment, osmotic stress, and chilling sensitivity [5]. A major source of bovine oocytes is from abattoir ovaries and the abattoir material is very heterogeneous, since oocytes are punctured from follicles of different sizes. Follicle diameter was widely used as a selection criterion and a relationship between follicle diameter and oocyte developmental competence was established in cattle [6]. The ability of oocytes to complete all stages of development and to support freezing was related to follicle size [7] and consequently to the size of the oocyte [8].

There are a number of problems associated with vitrification of oocytes at different meiotic stages of maturation. The degree of oocyte maturation, from the germinal vesicle stage to the meiosis II (MII) stage, affects survival post-vitrification [9]. A significant increase in the diploid cells after maturation was reported [10] when oocytes were vitrified before (0 h) or after 8 h of the onset of in vitro maturation compared to those vitrified 12 or 22 h after the onset of maturation. Cryopreservation of large numbers of oocytes within a short period of time, ease of manipulation during cryopreservation and recovery, effects of cumulus cells on oocyte survival, and elimination of potential hazards of contamination were goals that were achieved with a modified method of vitrification using nylon mesh [11].

Capsule Vitrification of bovine oocytes: implications of follicular size and sire on the rates of embryonic development.

The source of oocytes and semen on vitrification of large numbers of cumulus-intact bovine oocytes and IVF resulted in no early embryo development differences.

V. M. Anchamparuthy · A. Dhali · W. M. Lott · R. E. Pearson ·
F. C. Gwazdauskas (✉)
Department of Dairy Science,
Virginia Polytechnic Institute and State University,
Blacksburg, VA 24061-0315, USA
e-mail: gwaz@vt.edu

In vitro fertilization can be used to evaluate bull fertility [12]. Correlations of cleavage and embryo production rates (IVF outcome) to the fertility of bull semen were reported [13–15]. The estimated relative conception rate (ERCR) is a measure of the fertility of an individual sire and is predictable and repeatable over the productive life of a sire used in artificial insemination (AI) programs, if sufficient data have been collected [16]. The ERCR-predicted fertility value is a 70-d non return to estrus rate of an AI service relative to the service sires of herd mates.

The overall objectives were to test whether or not the source of oocytes and semen impacted vitrification of large numbers of cumulus-intact bovine oocytes and subsequent IVF and early embryo development to test procedures that may assist with assisted reproductive technologies in humans. The success of vitrification was determined by subjecting vitrified-warmed oocytes to IVF and comparing fertilization and subsequent embryo development to those of fresh oocytes.

Materials and methods

The ERCR was calculated by USDA and relative values were assigned to 4 bulls on the basis of the field fertility, non-return to estrus rate at 70 d after AI. Bull I was rated 1; Bull II was -3; Bull III was 2; and Bull IV was -3. The data were obtained from Dairy Records Management Systems (Raleigh, NC) as of February 2007.

Collection of germinal vesicle-cumulus oocyte complexes

Two to 10 mm sized follicles were aspirated from abattoir obtained ovaries (Brown Packing Co Inc., Gaffney, SC) of cows of unknown reproductive status with a 10 cc syringe and 20 gauge needle into 2 labeled 50 mL conical tubes. The oocytes from follicles of different diameters, small (≤ 4 mm) and medium (4 to 10 mm) size were isolated and collected separately.

In vitro production of embryos

Maturation of oocytes was performed in tissue culture medium (TCM-199, Gibco Life Technologies, Inc.; Grand Island, NY) supplemented with 0.02 U/mL FSH (Sigma-Aldrich, St Louis, MO), 0.01 U/mL LH (Sigma-Aldrich), 1 μ g/mL 17 β -estradiol (E2; Sigma-Aldrich), and 10% fetal calf serum (FCS; Gibco Life Technologies, Inc.) in a humidified atmosphere of 5% CO₂ in the air at 38.5°C for 24 h [17].

For IVF, frozen semen from 4 bulls that were classified according to their ERCR was used. Two straws of semen were thawed in a water bath at 37°C for 1 min. Motile

spermatozoa were separated by a modified swim-up technique [18] in synthetic oviduct fluid (SOF) supplemented with 25 mM HEPES (SOF-H), FCS (2%), and sodium pyruvate (0.1 mg/mL; Sigma-Aldrich). After washing and centrifugation (700 $\times$ g for 10 min), spermatozoa were resuspended to a final concentration of 1×10^6 /mL. Following maturation, oocytes were washed in SOF-H medium and placed into 47 μ L SOF for IVF (SOF-IVF) medium drop (10 oocytes in 1 μ L SOF-IVF per drop) supplemented with 10 μ g/mL heparin sodium sulfate (Sigma-Aldrich) and BSA (8 mg/mL; Sigma-Aldrich) and 2 μ L of the sperm cell suspension was added to each microdrop of SOF-IVF medium (final volume of 50 μ L and final concentration of 1×10^6 spermatozoa/mL [19].

After 18 h of the oocyte and sperm cell incubation in a humidified atmosphere (5% CO₂ in air at 38.5°C) presumptive zygotes were denuded of cumulus cells by vortexing in 2.0 mL SOF-H medium for 4 min. Embryos were washed three times in SOF-H, and twice in SOF for IVC (SOF-IVC) and transferred to 50 μ L SOF-IVC culture drops supplemented with 1 mg/mL BSA under mineral oil (Specialty Media, Lavellette, NJ) and cultured at 100% humidity in a gas phase of 5% CO₂, 5% O₂ and 90% N₂ at 38.5°C [20]. The cultured drops were supplemented with 4% FCS at 96 h post culture.

Vitrification protocol

Vitrification of oocytes was conducted using a nylon mesh holder (pore size 60 μ m; Sefar America Inc., NY) as described [11]. A stepwise vitrification procedure [21] was adopted using an ethylene glycol, Ficoll, and sucrose (EFS40) vitrification solution. Oocytes after 15 h of maturation were washed three times with SOF-H and exposed for 7 min to 100 μ L droplets of 10% (v/v) ethylene glycol, 4.5% (w/v) Ficoll-70 and 0.075 M sucrose in Ca⁺⁺ free modified Dulbecco's PBS. Next, oocytes were exposed for 2 min to 100 μ L droplets of 20% (v/v) ethylene glycol, 9.0% (w/v) Ficoll-70 and 0.15 M sucrose in PBS, and finally they were immersed for 1 min in 100 μ L droplets of 40% (v/v) ethylene glycol, 18% (w/v) Ficoll-70 and 0.3 M sucrose in PBS in 35 mm culture dishes. After equilibration, 20 to 25 oocytes were loaded onto the nylon mesh holder, which was placed on a single fold towel (Wausau paper, 22.9 cm $\times$ 25 cm, Harrodsburg Plant, Harrodsburg, KY) to remove excessive solution, immediately transferred to 2 mL pre-cooled cryovials and directly plunged into liquid nitrogen (LN2) within 40 to 60 sec. After 24 h of storage in LN2, the oocytes were ultra-rapidly warmed, by maintaining them in air for 5 sec and the cryoprotectants were removed in a stepwise manner. The nylon mesh was transferred from LN2 into 2.7 mL of the warm Ca⁺⁺ free PBS (37°C) in 20% FCS with a sequential

series of 0.5, 0.25 and 0.125 M sucrose dilutions and by placing in each solution for 1, 2, and 3 min, respectively, and finally transferring into PBS (37°C) for 3 min in culture dishes. After warming, the nylon mesh was transferred into SOF-H medium. The adhered oocytes were separated from the mesh by gently flushing the oocytes with SOF-H drawn out of the pipette repeatedly and all of the oocytes were washed three times before they were transferred into TCM-199 for further maturation.

Assessment of oocyte survival

Oocytes with homogenous ooplasm, intact membranes, and zona pellucida post-thaw were designated as living oocytes [22]. Survival of vitrified-warmed oocytes was evaluated after 18 h in fertilization medium, 9 h after thawing. Cleavage (22 to 24 h after IVF), morula, and blastocyst formation rates (day 9) were used to assess their developmental competence [2].

Statistical analysis

For experiments on the effect of vitrification on the developmental capacity of bovine oocytes and the sire influence on the post IVF outcome, data was analyzed by using the PROC MIXED in SAS (9.1). Cleavage and development rates were determined in this experiment. The effects included in the model were treatment (fixed effect), bull (fixed effect), follicle size (fixed effect), and the interactions between treatment and follicle size, sire and follicle size, sire and treatment, and sire by treatment by follicle size. Dependent variables were percentage of oocytes cleaved, percentage of morulae, and percentage of blastocysts. Percentage data was analyzed with and without the arcsine transformation to correct for problems of non-normality associated with the analysis of percentage data. Data is presented as least squares means $\pm$ SEM, and differences between treatments were considered significant at $P < 0.05$. Pearson and Rank correlations were conducted between ERCR estimates for the 4 bulls and the significant cleavage (vitrified and control) means. Similarly, correlations were estimated between ERCR and the significant blastocyst vitrified and control means, and the significant blastocyst means for oocytes from small and medium-sized follicles using the PROC CORR procedure of SAS.

Results

Morphological survival of oocytes and embryos

The survival of oocytes post-thaw and embryos after IVF was evaluated by morphological examination. The recovery

of oocytes from both small and medium-sized follicles from the vitrification media was 99.3% of 1,968 initially equilibrated. The post thaw recovery from the nylon mesh was 98.7%. The results indicated that the vitrification treatment did not affect the proportion of the oocytes with intact morphology after warming as the survival rate after warming was 98.9%. The similarity of morphology of cumulus cells post vitrification and warming to controls following nylon mesh vitrification is shown in Fig. 1. After IVF there was no difference ($P > 0.05$) observed in the fragmentation of embryos formed from the vitrified (9.7%; $n = 1,968$) versus control (9.3%; $n = 1,685$) oocytes based on their morphology.

Embryonic development

The developmental rates of embryos based on the total number of oocytes fertilized was significantly ($P < 0.05$) affected by treatment (vitrified vs. control). The overall cleavage rates were significantly different ($P < 0.05$) for the vitrified and control oocytes (39.1% vs. 58.5%). The rates of embryo development to the morulae (13.1% vs. 35.1%) and blastocysts (5.1% vs. 22.9%) were also significantly different ($P < 0.05$) between the vitrified and control groups, respectively.

Follicle size of the oocytes had a significant ($P < 0.05$) impact on the developmental rates of embryos from cleavage to the blastocyst stage. There was a 9.9% advantage in the cleavage rate for oocytes from medium-sized follicles compared to oocytes from small-sized follicles. While medium-sized follicle oocytes had an 11.8% advantage in the developmental rate to morula, the difference in the blastocyst rate was only 5.9% between the oocytes from medium- and small-sized follicles (Table 1).

There was no interaction between treatment and follicle size of oocytes for cleavage rate; however, morula and blastocyst rates were significantly ($P < 0.05$) different (Table 1). The rate of development to the morula was 9.6% for vitrified oocytes from small-sized follicles and 16.6% for vitrified oocytes from medium-sized follicles in contrast to 26.9%, and 43.4%, respectively, for oocytes from control small- and medium-sized follicles. The vitrified oocytes from small- and medium-sized follicles resulted in 3.2 and 7.0% blastocyst rates, respectively, and the corresponding rates in control oocytes from small- and medium-sized follicles were 18.9 vs. 26.9%.

Sire influence on the in vitro embryonic development of vitrified oocytes

The cleavage and subsequent in vitro embryonic developmental rates showed variations among individual bulls (Fig. 2). The overall cleavage rates for Bull I, II, III, and IV were 49.3, 55.0, 50.0, and 40.9%, respectively ($P < 0.05$).

Fig. 1 Morphology of oocytes at 15 h of maturation. **a** and **b** without vitrification, **c** and **d** vitrified (presence of intact cumulus cells). *Left* (100 $\times$), and *right* (40 $\times$) magnification. Both vitrified and non-vitrified oocytes had homogenous ooplasm, intact membranes and zona pellucida

Both morula and blastocyst stages of development were different ($P < 0.05$) among the bulls. The developmental rates to morulae were 26.4, 29.9, 23.4 and 16.7% for Bull I, II, III, and IV, respectively. The blastocyst developmental rates were 18.1, 17.9, 10.0, and 10.0%, respectively.

Treatment by sire interaction had a significant effect ($P < 0.05$) on the cleavage and blastocyst rate. Nonetheless, there was no significant interaction ($P > 0.05$) between sire and oocyte follicle size for cleavage, but there was significant interaction ($P < 0.05$) for blastocyst development (Table 2). The sire by treatment by follicle size interaction had no impact ($P > 0.05$) on the development of the embryos.

Treatments significantly influenced cleavage and blastocyst developmental rates for Bull I. The cleavage rates were 39.9 and 58.7%, respectively, for the vitrified and control oocytes (Table 2). Bull I had the highest blastocyst develop-

ment for vitrified oocytes (10.9%) and the corresponding rate for the control oocytes was 25.3%. Additionally, there was a significant effect ($P < 0.05$) by the size of the follicle source on oocytes for the development to the blastocyst. The oocytes derived from small-sized follicles developed to 15.2% blastocyst in contrast to 20.9% for those derived from medium-sized follicles.

The Bull II had the highest cleavage rates for the control oocytes (69.3%). On the other hand, the cleavage rate of the vitrified oocytes using Bull II for IVF showed a pattern of development similar to that of Bull I, 40.8%; however the blastocyst development rate for the vitrified oocytes had a lower developmental pattern (6%). Bull II showed highest blastocyst (23.1%) development for the oocytes derived from medium-sized follicles, but the development for those derived from smaller follicles was lower (12.8%) than Bull I.

Table 1 Least squares means for embryonic developmental rate (%) $\pm$ SE of oocytes from small and medium follicles, and interactions with vitrified vs. control oocytes

Treatment	Cleavage			Morula			Blastocyst		
	Medium	Small	Difference	Medium	Small	Difference	Medium	Small	Difference
Size*	53.7 ^a $\pm$ 1.56	43.8 ^b $\pm$ 1.59	9.9	30.0 ^a $\pm$ 1.75	18.2 ^b $\pm$ 1.78	11.8	16.9 ^a $\pm$ 1.00	11.0 ^b $\pm$ 1.15	5.9
Vitrified	42.8 $\pm$ 1.56	35.4 $\pm$ 1.61	7.4 ^c	16.6 $\pm$ 1.75	9.6 $\pm$ 1.80	7.0	7.0 $\pm$ 1.00	3.2 $\pm$ 1.03	3.8
Control	64.7 $\pm$ 1.56	52.3 $\pm$ 1.56	12.4	43.4 $\pm$ 1.75	26.8 $\pm$ 1.75	16.6	26.9 $\pm$ 1.00	18.9 $\pm$ 1.00	8.0

^{a,b} Means with different superscripts within the row are different at $P < 0.05$. ^c Non-significant interaction

*significant main effect ($P < 0.05$)

Fig. 2 Sire influence on the in vitro development of embryos, developmental rate $\pm$ SE (%)

The influence of Bulls III and IV for the first division following fertilization in both vitrified and control oocytes was similar to that of Bulls I and II, but further development was further reduced in both bulls for both vitrified and control oocytes.

A reduction in the blastocyst rates was observed in both Bull III and Bull IV for the vitrified and control oocytes. Bull III had 2.5 vs. 17.4% blastocysts in vitrified and control oocytes, respectively, in contrast to 1 vs. 19.0% obtained in Bull IV. The significant impact of follicle size on blastocyst development in the Bull III and Bull IV was similar for both sizes. Oocytes from small-sized follicle had blastocyst rates of 8.3 and 7.9% in contrast to 11.7 and 12.0%, respectively, for the oocytes from medium-sized follicles (Table 2). Our study did not observe overall correlations between ERCR estimates of the 4 tested bulls and the developmental rates of embryos.

Discussion

Effect of vitrification on bovine oocytes

Oocyte cryopreservation using nylon mesh vitrification resulted in high oocyte recovery, post warming survival, fertilization and blastocyst development. Vitrification and recovery of large numbers of cumulus-intact bovine oocytes

in a closed system was successful. Yet, the overall cleavage and blastocyst rates were significantly lower for the vitrified oocytes than controls (Table 1). Only 2 studies have used nylon mesh for vitrification of germinal vesicle bovine oocytes [11, 21]. In our study using Bull I for IVF resulted in the vitrified oocytes cleaving (39.9%) and developing into morula (18.4%) and blastocysts (10.9%) at a higher rate than other bulls. Still, cleavage rate and morulae and blastocysts yields were below that of control oocytes (58.7, 34.4, and 25.3%, respectively). We found increased developmental rates of embryos in contrast to others [11, 21] using nylon mesh. The cooling rate using pre-cooled cryovials for vitrification might have had beneficial effects on the post thaw survival and embryonic development. Similar blastocyst rates were reported with matured oocytes (MII) that were vitrified with the cryoloop (10%) [23, 24], but a lower rate was reported for OPS (5%) [24] and conventional straws (4.8%) [25] using MI stage oocytes.

Although we have not determined the cooling and warming rates, both are assumed high, because of the usage of pre-cooled cryovials, easy handling of nylon mesh for mounting and locating oocytes, and recovery of normal oocytes with little damage (Fig. 1; Table 1). The presence of cumulus cells surrounding the oocytes is crucial for ensuring complete oocyte maturation and for developmental competence [26]. Yet, the unequal thickness and density of the cumulus cells may result in differences in cryoprotectant penetration. Nevertheless, cumulus cells are beneficial for protecting the oocytes against osmotic shock caused by rapid concentration or dilution of cryoprotectants and by providing a rigid structure around oocytes that prevents its morphological damage [27]. Our data supports these findings. A striking feature of our study was the retention of the cumulus cells after rewarming (Fig. 1). Furthermore, 6 to 8 h of maturation is the stage at which the cumulus cells act as a crucial supportive and regulatory role in RNA synthesis [28].

Follicle size had a significant impact on the development of embryos. Oocytes from small follicles (≤ 4 mm) developed at lower rates than oocytes from medium (4 to 10 mm) sized follicles whether or not they were undergoing vitrification or were just simply acting as controls. Follicle diameter and atresia, cumulus oocyte complex morphology,

Table 2 Least squares means (%) $\pm$ SE for significant ($P < 0.05$) interactions of sire by treatment and sire by source of oocytes

Sire No.	Cleavage		Blastocyst		Blastocyst	
	Vitrified	Control	Vitrified	Control	Medium	Small
1	39.9 $\pm$ 2.2	58.7 $\pm$ 2.0	10.9 $\pm$ 1.5	25.3 $\pm$ 1.4	20.9 $\pm$ 0.01	15.2 $\pm$ 0.02
2	40.8 $\pm$ 2.4	69.3 $\pm$ 2.4	6.0 $\pm$ 1.7	29.9 $\pm$ 1.7	23.1 $\pm$ 0.02	12.8 $\pm$ 0.02
3	39.4 $\pm$ 3.9	60.4 $\pm$ 2.4	2.5 $\pm$ 1.7	17.4 $\pm$ 1.7	11.7 $\pm$ 0.02	8.3 $\pm$ 0.02
4	36.2 $\pm$ 2.36	45.5 $\pm$ 2.4	1.0 $\pm$ 1.7	19.0 $\pm$ 1.7	12.0 $\pm$ 0.02	7.9 $\pm$ 0.02

and oocyte diameter were the important non-invasive quality markers for oocyte competence. A clear correlation between follicle diameter and oocyte developmental competence was established in cattle [6]. In cattle, follicles with <3 mm diameters contain oocytes with a diameter under 110 μm , which are considered developmentally incompetent [29] and the proportion of competent oocytes increased greatly in follicles >8 mm diameter [30]. None of the studies reported were directed toward the cryotolerance potential of oocytes from different follicle sizes. Our results showed that 35.4, 9.6, and 3.2% vitrified oocytes from small-sized follicles were able to cleave and yield morulae and blastocysts, respectively, in contrast to 42.8, 16.6 and 7% of vitrified oocytes from medium-sized follicles. There was an interaction for morula and blastocyst development, showing a greater rate of decline in development for oocytes from small follicles compared to oocytes from medium-sized follicles.

Sire influence on in vitro embryonic development

The influence of sires on the in vitro cleavage, development to blastocyst of vitrified and control oocytes, and blastocyst development from small- and medium-sized follicles was revealed. A marked difference among bulls at the blastocyst stage development was recorded in earlier in vitro studies [14, 20]. Variations in IVF characteristics among bulls were reported [20], but this is the first reported with vitrification of oocytes. Individual batches of bovine oocytes fertilized with the semen of different bulls vary between 0 and 36% in their capacity to produce embryos in vitro [18, 31]. Bulls of high fertility produced embryos that were more likely to develop to the blastocyst stage than bulls of lower fertility. Bulls can differ in embryo generating capacities, even if they display the same fertilization in vitro [32].

The ERCR is a phenotypic predictor of bull fertility, expressed as relative conception rate. It is estimated that a Bull with +2 or higher ERCR value would have an impact on fertility [33]. Bull I with +1 ERCR and Bull II with -3 ERCR showed higher cleavage and blastocyst rates than Bulls III and IV. Bull IV, with -3 ERCR, had a lower cleavage and blastocyst rate development than other bulls. Bull III with +2 ERCR showed a higher cleavage rate than Bull IV, but a similar blastocyst rate. Cornwell et al. [34] showed a high deviation in the ERCR values from time to time.

Although a correlation between IVF outcomes and bull fertility was found in the previous studies [13, 14], our study did not observe correlations between ERCR estimates of the 4 tested bulls and the developmental rates of embryos. The discrepancy may be due to differences among bulls in fertilization rates [13] and in embryo cleavage and development to the morula and blastocyst stages [35]. We found little evidence that post IVF outcome

provided a useful predictor of field fertility of bulls. Final conclusions on the usefulness of in vitro results will require work on a larger scale. Our study showed that nylon mesh vitrification resulted in high survival of vitrified oocytes, and allowed a reliable recovery of oocytes from liquid nitrogen. Follicle size for oocytes had a direct impact on the success of vitrification of oocytes as did the sire used for IVF. Additionally, no relation existed between post-IVF outcome and field fertility based on ERCR.

Toward this end, it is noteworthy that vitrification is beginning to enter the main stream of human assisted reproductive technology programs based on the protocols successfully applied to bovine oocytes and embryos. One of the important elements of quality control in stimulation for IVF in humans is follicular status. Although several human studies have suggested oocytes derived from larger follicles outperform oocytes originating from smaller follicles, correlation of cryotolerance with follicular size has not been characterized. This study enabled us to demonstrate potential outcomes of vitrification based on follicular size of oocytes. As bovine oocytes are more sensitive to the effects of cooling than human oocytes, the methods developed in the bovine should produce better results when adapted for humans. We suggest including follicle size of oocytes as a criterion in qualifying human follicles and oocytes for cryopreservation rather than subjecting the entire cohort to cryopreservation.

References

1. Leibo SP. Water permeability and its activation energy of fertilized and unfertilized mouse ova. *J Membr Biol.* 1980;53:179–88.
2. Men H, Monson RL, Rutledge JJ. Effect of meiotic stages and maturation protocols on bovine oocyte's resistance to cryopreservation. *Theriogenology.* 2002;57:1095–103.
3. Vajta G, Holm P, Kuwayama M, Booth PJ, Jacobsen H, Greve T, et al. Open Pulled Straw (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos. *Mol Reprod Dev.* 1998;51:53–8.
4. Dhali A, Anchamparathy VM, Butler SP, Pearson RE, Mullarky IK, Gwazdauskas FC. Gene expression and development of mouse zygotes following droplet vitrification. *Theriogenology.* 2007;68:1292–8.
5. Massip A. Cryopreservation of bovine oocytes: current status and recent developments. *Reprod Nutr Dev.* 2003;43:325–30.
6. Fuhrer F, Mayr B, Schellander K, Kalat M, Schleger W. Maturation competence and chromatin behaviour in growing and fully grown cattle oocytes. *Zentralbl Veterinarmed A.* 1989;36:285–91.
7. Lonergan P, Monaghan P, Rizos D, Boland MP, Gordon I. Effect of follicle size on bovine oocyte quality and developmental competence following maturation, fertilization, and culture in vitro. *Mol Reprod Dev.* 1994;37:48–53.
8. Fair T, Hyttel P, Greve T. Bovine oocyte diameter in relation to maturational competence and transcriptional activity. *Mol Reprod Dev.* 1995;42:437–42.
9. Parks JE, Ruffing, NA. Factors affecting low temperature survival of mammalian oocytes. *Theriogenology.* 1992;59–75.

10. Luna HS, Ferrari I, Rumpf R. Influence of stage of maturation of bovine oocytes at time of vitrification on the incidence of diploid metaphase II at completion of maturation. *Anim Reprod Sci.* 2001;68:23–8.
11. Matsumoto H, Jiang JY, Tanaka T, Sasada H, Sato E. Vitrification of large quantities of immature bovine oocytes using nylon mesh. *Cryobiology.* 2001;42:139–44.
12. Gordon I, Lu KH. Production of embryos in vitro and its impact on livestock production. *Theriogenology.* 1990;77–87.
13. Marquant-Le Guenne B, Humblot P, Thibier M, Thibault C. Evaluation of bull semen fertility by homologous in vitro fertilization tests. *Reprod Nutr Dev.* 1990;30:259–66.
14. Shamsuddin M, Larsson B. In vitro development of bovine embryos after fertilization using semen from different donors. *Reprod Dom Anim.* 1993;77–84.
15. Lonergan P. The application of in vitro fertilization techniques to the prediction of bull fertility. *Reprod Dom Anim.* 1994;12–21.
16. Clay JS, McDaniel BT. Computing mating bull fertility from DHI nonreturn data. *J Dairy Sci.* 2001;84:1238–45.
17. Walters AH, Eyestone WE, Saacke RG, Pearson RE, Gwazdauskas FC. Bovine embryo development after IVF with spermatozoa having abnormal morphology. *Theriogenology.* 2005;63:1925–37.
18. Parrish JJ, Susko-Parrish JL, Leibfried-Rutledge ML, Critser ES, Eyestone WH, First NL. Bovine in vitro fertilization with frozen-thawed semen. *Theriogenology.* 1986;25:591–600.
19. Parrish JJ, Krogenaes A, Susko-Parrish JL. Effect of bovine sperm separation by either swim-up or Percoll method on success of in vitro fertilization and early embryonic development. *Theriogenology.* 1995;44:859–69.
20. Ward F, Rizos D, Corridan D, Quinn K, Boland M, Lonergan P. Paternal influence on the time of first embryonic cleavage post insemination and the implications for subsequent bovine embryo development in vitro and fertility in vivo. *Mol Reprod Dev.* 2001;60:47–55.
21. Abe Y, Hara K, Matsumoto H, Kobayashi J, Sasada H, Ekwall H, et al. Feasibility of a nylon-mesh holder for vitrification of bovine germinal vesicle oocytes in subsequent production of viable blastocysts. *Biol Reprod.* 2005;72:1416–20.
22. Men H, Monson RL, Parrish JJ, Rutledge JJ. Detection of DNA damage in bovine metaphase II oocytes resulting from cryopreservation. *Mol Reprod Dev.* 2003;64:245–50.
23. Luster SM. Cryopreservation of bovine and caprine oocytes by vitrification. Louisiana: Louisiana State University; 2004.
24. Checura CM, Seidel GE Jr. Effect of macromolecules in solutions for vitrification of mature bovine oocytes. *Theriogenology.* 2007;67:919–30.
25. Hochi S, Ito K, Hirabayashi M, Ueda M, Kimura K, Hanada A. Effect of nuclear stages during IVM on the survival of vitrified-warmed bovine oocytes. *Theriogenology.* 1998;49:787–96.
26. Fukui Y, Sakuma Y. Maturation of bovine oocytes cultured in vitro: relation to ovarian activity, follicular size and the presence or absence of cumulus cells. *Biol Reprod.* 1980;22:669–73.
27. Park SE, Chung HM, Cha KY, Hwang WS, Lee ES, Lim JM. Cryopreservation of ICR mouse oocytes: improved post-thawed preimplantation development after vitrification using Taxol, a cytoskeleton stabilizer. *Fertil Steril.* 2001;75:1177–84.
28. Gordon I. Laboratory production of cattle embryos. 2nd ed. Walingford: CABI; 2003. p. 278. CAB International.
29. Gandolfi TA, Gandolfi F. The maternal legacy to the embryo: cytoplasmic components and their effects on early development. *Theriogenology.* 2001;55:1255–76.
30. Hendriksen PJ, Vos PL, Steenweg WN, Bevers MM, Dieleman SJ. Bovine follicular development and its effect on the in vitro competence of oocytes. *Theriogenology.* 2000;53:11–20.
31. Lacalandra GM, Dell'Aquila ME, Fusco S, Sciorci RL, Perrone P, Minoia P. In vitro fertility test of bull semen. Sydney: The 13th International Congress on Animal Reproduction; 1993.
32. van Soom A, Ysebaert MT, de Kruif A. Relationship between timing of development, morula morphology, and cell allocation to inner cell mass and trophectoderm in in vitro-produced bovine embryos. *Mol Reprod Dev.* 1997;47:47–56.
33. Pursley R. Seven surefire ways to improve fertility of dairy cows. Michigan: Michigan Dairy Rev; 2004. p. 1–2.
34. Cornwell JM, McGilliard ML, Kasimanickam R, Nebel RL. Effect of sire fertility and timing of artificial insemination in a Presynch + Ovsynch protocol on first-service pregnancy rates. *J Dairy Sci.* 2006;89:2473–8.
35. Zhang BR, Larsson B, Lundeheim N, Rodriguez-Martinez H. Relationship between embryo development in vitro and 56-day nonreturn rates of cows inseminated with frozen-thawed semen from dairy bulls. *Theriogenology.* 1997;48:221–31.