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Original article

Cryopreservation of bovine oocytes: is cryoloop vitrification the future to preserving the female gamete?

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Abstract — The cryoloop is a technique where a thin nylon loop is used to suspend a film of cryoprotectant containing the oocytes and directly immersing them in liquid nitrogen. 508 bovine oocytes were collected, of these 351 were cryopreserved by slow freezing using standard straws or a new vitrification method using our self-constructed cryoloops and the remainder were controls. After thawing, the oocytes were inseminated by ICSI or standard IVF. The cryoloop vitrification method yielded a survival rate of 90.5% and the slow freezing technique a rate of 54.4% ($p < 0.0001$). When ICSI was performed, cryopreservation by the cryoloop vitrification method resulted in very similar cleavage rate to controls (16.0% vs. 17.3%) but slow freezing produced a slightly lower rate (9.4%). Cleavage rates after IVF in fresh oocytes was higher than the cryopreservation groups (49.5% vs. 15.4% and 25.8%), whereas after ICSI the rates were similar in all groups (17.3% vs. 9.4% and 16%). It is concluded that the new cryoloop vitrification technique followed by ICSI produce good embryo formation results and they could hold the future for effective oocyte cryopreservation.

bovine / cryoloop / ICSI / oocyte / vitrification

1. INTRODUCTION

Cryopreservation of oocytes is one of the most promising options of the future in human female gamete preservation and donation. However the survival rates and fertilisation potential of the cryopreserved oocytes are currently very small. Several healthy children were born as a result of this procedure so far [7, 19], but it is too soon for reliable application of the technique routinely [16, 17].

Unlike sperm and embryos, oocytes are very vulnerable to the procedure

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of cryopreservation. Vitrification can be achieved with short exposure to high concentrations of cryoprotectants followed by very fast cooling and warming rates [5] in order to avoid crystallisation damage. Using standard freezing straws and cryovials or even open-pulled straws [6], actual vitrification of the medium containing the oocytes is not always successful due to the delay of heat loss from the medium [15]. This causes crystallisation, which is detrimental to the oocyte. In the slow freezing technique the main aims are to equilibrate the cells with the cryoprotectant and minimise the formation of large ice crystals by seeding at subzero temperatures [9, 12] and addition of non-permeable cryoprotectants such as sucrose [2].

The cryoloop, used as a vessel in vitrification, is a thin nylon loop used to suspend a film of cryoprotectant containing the oocytes and directly immerse them in liquid nitrogen. So far, the cryoloop has been used on human, mouse [8] and monkey [18] blastocysts with excellent survival rates and no difference in pregnancy rates (mouse) compared to controls. The aim of this study was to evaluate the novel cryoloop vitrification method for the cryopreservation of bovine oocytes, followed by IVF and ICSI.

2. MATERIALS AND METHODS

2.1. Oocyte preparation

Needle aspiration of antral follicles of abattoir-derived bovine ovaries transported within four hours of slaughtering and stored in saline and antibiotics at 39 °C took place. Cumulus-oocyte complexes (COC's) were cultured in serum-free defined culture medium based on Dulbecco's modified essential medium with Ham's F12 (DMEM: F12, Sigma) supplemented with ovine luteinising hormone (oLH) at 100 ng·mL^{-1} at 37 °C and in 5% CO_2 for about 26 hours. The COC's were exposed to hyaluronidase 400 iu·mL^{-1} (Sigma) for not more than 7 min during which the cumulus cells were removed mechanically from the oocytes destined for ICSI and one or two layers of cumulus cells left attached on those oocytes destined for IVF. 508 oocytes were randomly allocated into three groups; controls, slow freezing and cryoloop vitrification. The maturational stage of the oocytes was not established due to the presence of cumulus cells (IVF group) and intracytoplasmic lipid obstructing microscopic view. Separate experiments were performed for IVF and ICSI due to inadequate oocyte numbers in each run.

2.2. Vitrification

The selected oocytes for vitrification were exposed to 20% Foetal Calf Serum (FCS) in HEPES Synthetic Oviductal Fluid (SOF) for about 20 min at room temperature (24 °C). Then they were placed in V1 (10% FCS, 10% Dimethyl sulfoxide (ME2SO), 10% Ethylene Glycol (EG), 1 mg·mL^{-1} Polyethylene Glycol (PEG) in Phosphate Buffered Saline (PBS), all by Sigma) for about 20 s at room temperature (Figs. 1A and 1B) and then transferred to V2 (10% FCS, 20% ME2SO, 20% EG, 10 mg·mL^{-1} Ficoll (MW 400 000), 0.65 M sucrose, 1 mg·mL^{-1} PEG in PBS, all by Sigma) and placed onto a cryoloop pre-loaded with a thin film of V2 (Fig. 2) by a finely drawn glass pipette powered by the forces of surface tension exposing them to V2 for a total of 20 s at room temperature (Fig. 1C). The self-constructed cryoloop was made from 80 μm thick nylon forming a loop of 1 mm diameter mounted on a plastic pipette tip and the inner surface of the lid of a 4 mL cryovial. The loaded cryoloop was plunged into liquid nitrogen and then screwed on the cryovial already immersed in liquid nitrogen. Standard canes were used for storage.

For warming, the cryoloop was removed from the liquid nitrogen and placed on top of thawing medium VT1 (10% FCS, 1 M sucrose in PBS, Sigma chemicals) at 37 °C

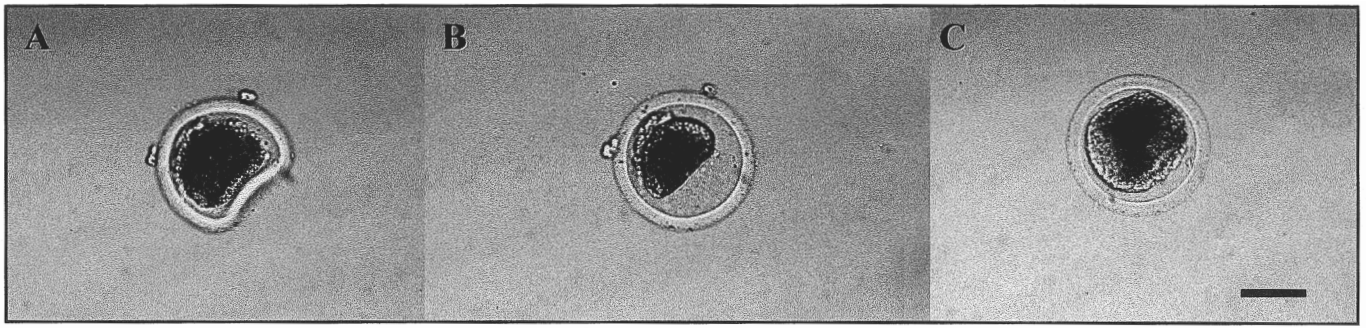

Figure 1. Oocyte changes after exposure to vitrification media. Immediately after exposure to V1 (**A**) there is sudden dehydration and deformation, 20 s in V1 (**B**), the medium starts to diffuse through the zona pellucida into the perivitelline space, and finally an additional 20 s in V2 (**C**), the medium diffuses into the ooplasm causing partial re-hydration. Bar 0.06 mm.

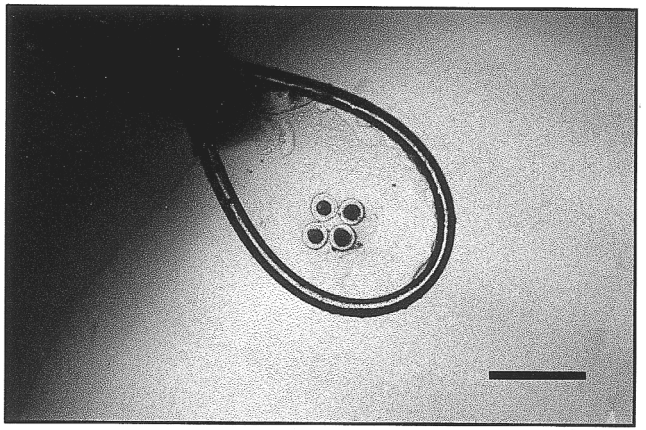

Figure 2. The loaded cryoloop showing the oocytes suspended in a thin film of vitrification medium held by the force of surface tension. Bar 0.5 mm.

and as soon as the cryoloop contents liquefied, the loop was immersed in the VT1 drop. The oocytes were drawn by surface tension forces, and were transferred sequentially to VT2 (10% FCS, 0.5 M sucrose in PBS, Sigma), VT3 (10% FCS, 0.25 M sucrose in PBS, Sigma) and finally VT4 (10% FCS, 0.125 M sucrose in PBS, Sigma) being exposed for 2.5 min in each medium at 37 °C. After washing three times in DMEM:F12 with HEPES (Sigma) they were cultured in the same medium for at least one hour.

2.3. Slow-freezing

Oocytes were exposed to 20% FCS in HEPES SOF for about 20 min, transferred in F1 (10% FCS, 1.5 M 1,2-propanediol (PROH) in PBS, Sigma) for 15 min and then in F2 (10% FCS, 1.5 M PROH, 0.1 M sucrose in PBS, Sigma) for 5 min, all at room temperature. A 0.25 mL cryopreservation straw was used and about 0.07 mL of F2 was aspirated first, followed by 0.07 mL of F2 containing the oocytes until the first column reached the cotton wool stopper. The two columns were separated by a small air gap and the end sealed by a plastic plug. The straws were placed in the programmable PLANER® freezer and the cooling program started as follows: Start at 23 °C, –2 °C per min to –8 °C and held at this temperature for 5 min, –0.3 °C per min to –30 °C, –50 °C per min to –140 °C and held at this temperature for 15 min. At the temperature of –8 °C, seeding took place by touching the straws at the level of the second column with sponge-holding forceps at –196 °C. Seeding was successful when formation of ice crystals was observed within the clear medium inside the straw. At the end of the program at –140 °C, the straws were removed and immediately plunged into liquid nitrogen for storage.

For thawing, each straw was placed in a water bath at 30 °C until the crystals dissipated and the contents emptied in a Petri dish. Sequential transfer then took place in thawing media FT1 (10% FCS, 1.0 M PROH, 0.2 M sucrose in PBS, Sigma), FT2 (10% FCS, 0.5 M PROH, 0.2 M sucrose in PBS, Sigma), FT3 (10% FCS, 0.25 M PROH, 0.2 M sucrose in PBS, Sigma) and FT4 (10% FCS, 0.2 M sucrose in PBS, Sigma), exposing the oocytes for 2.5 min in each medium at 37 °C. After washing three times in DMEM:F12 with HEPES (Sigma) they were cultured in the same medium for at least one hour.

2.4. Fertilisation and embryo culture

For intracytoplasmic sperm injection (experiment 1), a motile spermatozoon was identified, immobilised and injected into the ooplasm using an inverted microscope (Olympus IMT-2) with a micromanipulation system (Research Instruments, Penryn, Cornwall, UK). For in vitro fertilisation (experiment 2), oocytes were inseminated with thawed bull's sperm at a concentration of $300\,000 \cdot mL^{-1}$ and left in the incubator for not more than 20 hours. Synthetic Oviductal Fluid (SOF) supplemented with amino acids and bovine serum albumin (SOFaaBSA) was prepared and used for the culture of fertilised oocytes. After all the oocytes were either injected (experiment 1) or inseminated (experiment 2) they were washed twice in HEPES SOF medium, once in SOFaaBSA medium and then transferred in SOFaaBSA 50 μL culture drops under mineral oil. 48 hours later, they were transferred to fresh drops of SOFaaBSA and embryo growth was observed and recorded.

2.5. Main outcome measures

Oocytes with fragmented cytoplasm, indistinct oolemma, increased perivitelline space or damaged zona pellucida at 2 hours after thawing were considered as non-survivors. Morphologically normal oocytes that degenerated after at least 48 hours of culture were either damaged during ICSI or

had not survived cryopreservation. Therefore, the number of oocytes that degenerated during in vitro culture was recorded as a separate group from non-survivors 2 hours after thawing.

2.6. Statistical analysis

Binomial statistics were performed assuming normal distribution where sample number was greater than 30. A χ^2 test was performed on the groups using GraphPad Prism™ (version 3.0) software to obtain a *p*-value. Values of *p* less than 0.05 were considered statistically significant at the 95% confidence level.

3. RESULTS

Table I compares the two techniques of cryopreservation showing similar oocyte recovery rates at high rates in excess of 95%. "Lost" oocytes dropped off the cryoloop, attached themselves inside the cryo-straw or were misplaced due to operator error. Oocyte survival was significantly better in the vitrification group at 90.5% compared to slow-freezing with 54.4%.

In the ICSI subset, oocyte degeneration following at least 48 hours of culture was significantly higher in the slow freezing group compared to controls and the cryoloop vitrification group. Cleavage rates after ICSI were similar between the control and cryoloop vitrification groups and a lower trend in the slow freezing group (Tab. II) compared to controls was observed.

In the IVF subset, oocyte degeneration was higher in the slow freezing group compared to controls and the cryoloop vitrification group. The cleavage rate after IVF was much higher in fresh oocytes than the slow freezing and vitrification groups (Tab. II). The collected oocytes destined for each control and study subgroup was recorded and enabled us to determine the overall embryo formation rate, from oocyte collection to oocyte cleavage (Tab. II).

4. DISCUSSION

This study has shown that embryo formation two days after insemination following cryopreservation of bovine oocytes using the cryoloop vitrification method and ICSI is not different from that obtained using fresh oocytes. The fact that the embryo formation rates are lower in the ICSI subset, compared to the IVF subset, is due to failure of activation of the bovine oocytes by the ICSI procedure itself and this has been shown in previous studies. The use of activation agents was not appropriate in this study due to a high rate of parthenogenetic activation. The purpose of this study was to provide means of bypassing the zona pellucida barrier, which is responsible for a reduction in embryo formation after cryopreservation and standard IVF. The absence of a difference in embryo formation between fresh and cryopreserved oocytes is more

Table I. Oocyte recovery and survival rates of the two techniques of cryopreservation.

Group	Cryopreserved	Recovered		Survived (2 h post-thawing)	
		n	% ± SD	*n*	% ± SD
Slow freezing	189	180	95.2 ± 1.5	98	54.4 ± 3.7*
Vitrification	162	158	97.5 ± 1.2	143	90.5 ± 2.3*

* $p < 0.0001$, SD = standard deviation.

Table II. Oocyte degeneration and cleavage rates following slow-freezing or vitrification and IVF or ICSI.

Insemination method	Group	Inseminated survivors	Degeneration after insemination		Cleavage		Overall embryo formation (collection to cleavage)	
			n	% ± SD	*n*	% ± SD	*n*	% ± SD
ICSI	Control	52	8/52	15.4 ± 5.0[b]	9/52	17.3 ± 5.2[f]	9/52	17.3 ± 5.2[j]
	Slow freezing	53	24/53	45.3 ± 6.8[b, c]	5/53	9.4 ± 4.0[m]	5/109	4.6 ± 2.0[j, k]
	Vitrification	75	21/75	28.0 ± 5.2[c]	12/75	16.0 ± 4.2[n]	12/87	13.8 ± 3.7[k]
IVF	Control	105	9/105	8.6 ± 2.7[a]	52/105	49.5 ± 4.9[d, e, f]	52/105	49.5 ± 4.9[g, h]
	Slow freezing	39	12/39	30.8 ± 7.4[a]	6/39	15.4 ± 5.8[d]	6/80	7.5 ± 2.9[g, i]
	Vitrification	62	10/62	16.1 ± 4.7[l]	16/62	25.8 ± 5.6[e]	16/75	21.3 ± 4.7[h, i]

[c] $p < 0.05$; [d, e, i, j, k] $p < 0.01$; [a, b] $p < 0.001$; [f, g, h] $p < 0.0001$; [l, m, n] no significant difference to comparable groups; SD = standard deviation.

significant than the embryo formation rate per se.

In experiment 1 (ICSI) cryopreservation by the cryoloop vitrification method has resulted in very similar cleavage rate to controls. This observation implies that the vitrified/warmed oocytes, which have been selected as survivors have similar fertilisation potential as the healthy looking fresh ones when ICSI is undertaken. It may be argued that the cryopreservation procedure can induce parthenogenic activation and reduce fertilisation at the same time, in this way one effect increasing and the other decreasing the true cleavage rate, resulting in this apparent similarity in cleavage rates. It has been shown previously that parthenogenic activation rates in human [3] and bovine [1] oocytes do not increase after cryoprotectant exposure or cooling.

In experiment 2 (standard IVF), the cleavage rate in controls was much higher than ICSI, but when cryopreserved oocytes were fertilised the rates were lower in each group. The observation here is that cleavage rates are similar between study groups in ICSI but very different when IVF is used. The most probable reason for this is the effect of cryopreservation on zona pellucida hardening, which has been previously proven by molecular studies [4, 11, 13]. Oocyte degeneration was higher in the slow-freezing group implying occult damage, caused by this method of cryopreservation, escaping the initial inspection for survival and this was an observation in both experiments.

Previous studies on mature bovine oocytes have shown good survival rates using conventional vitrification methods [14] but failed to demonstrate an adequate embryo formation rate after IVF probably due to the effects of zona hardening and occult damage in the oocyte's sensitive structure.

Cryopreservation causes hardening of the zona pellucida leading to reduced fertilisation rates, making ICSI the preferred method of insemination. The evidence for the advantage of this method over standard IVF is strong enough to employ it as a routine method for insemination. Human oocytes become activated by the ICSI procedure and were they to show the same survival and cleavage rates following cryoloop vitrification it would be a possible emerging way of preserving the human oocyte. However, certain aspects should be further evaluated. There is room for improvement of in-vitro-maturation efficiency since this is a major determining factor for oocyte cleavage rates. Previous published data has shown fresh oocyte IVF cleavage rates of up to 80% [10]. An appropriate future study would be to evaluate the cryoloop vitrification technique in mature mouse oocytes. The advantage of this study would be that oocyte maturity would occur in vivo and simulate the human assisted reproduction procedures. What is more, murine oocytes would also get activated when injected during ICSI, producing in this way a higher number of embryos. The next step would be to apply the technique to human oocytes, but avoidance of infectious contamination by direct exposure to liquid nitrogen is of paramount importance.

It is concluded that it is possible to cryopreserve oocytes using the new cryoloop vitrification technique followed by ICSI with high rates of survival and similar cleavage rates to fresh oocytes. Once the overall embryo formation rates improve the above technique may potentially be the key to preserving the female gamete and replace, in some cases, embryo cryopreservation and frozen embryo transfer.

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