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Vitrification of immature and matured bovine oocytes: effect of brilliant cresyl blue selection and hyaluronan addition

P. Rodriguez-Villamil[1], F.L. Ongaratto[1], G. Moreira[1], M. Fernandez Taranco[1], G.A. Bó[1,2,3]

[1]Instituto de Reproducción Animal Córdoba (IRAC), Córdoba, Argentina.
[2]Instituto de Ciencias Básicas y Aplicadas, Universidad Nacional de Villa María, Córdoba, Argentina.

Abstract

The aim of the present study was to evaluate the effect of brilliant cresyl blue (BCB) selection, the type of oocyte (immature or matured) and the use of hyaluronan in the vitrification solution on further embryo developmental competence. Oocytes (n = 1308) obtained from abattoir ovaries were classified by BCB stain. Control oocytes were maintained in holding media for 90 min and then subdivided to be placed into maturation media without any treatment or were vitrified. Immature or matured oocytes were vitrified by the solid-surface technique using two different vitrification solutions. VS1: composed of 10% ethylene glycol (EG) for 10 min followed by 20% EG + 0.2M trehalose for 30 sec and finally into 30% EG + 0.5M trehalose for 30 sec, or VS2 composed by 10% EG for 10 min, followed by 20% EG + 0.2M trehalose for 30 sec, and finally into 30% EG+ 0.5M trehalose + 0.1 g/ml hyaluronan for 30 sec. Oocytes were then loaded into Fyberplugs™ and vitrified. After one week, Fyberplugs™ were open and placed directly into (37°C) 0.5M sucrose solution for 5 min, then into 0.25M of sucrose for another 5 min and finally placed into maturation medium for *in vitro* production. Cleavage and development rates were examined on days 2 and 7 after fertilization, respectively. The blastocyst rate of vitrified oocytes selected as BCB + (5.5 ± 0.6%) were higher than those selected as BCB - (1.0 ± 0.4%) and those that were not selected by BCB (2.0 ± 1.1%; $P < 0.001$). Furthermore, immature vitrified oocytes had greater ($P < 0.05$) cleavage and blastocyst rates (44.8 ± 1.9% and 4.0 ± 0.6%) than matured vitrified oocytes (38.3 ± 2.8% and 2.5 ± 0.6%). Finally, the addition of hyaluronan to the vitrification solution had no significant effect on development rates. In conclusion, the selection of oocytes by BCB and the use of immature oocytes increase the development rates of vitrified-warmed oocytes.

Keywords: BCB, cryopreservation, hyaluronan, oocyte selection.

Introduction

Cryopreservation of semen and embryos nowadays is used successfully for the preservation and widespread distribution of animal genetics around the world. However, oocytes are much more difficult to cryopreserve than embryos due to their morphology, higher cryosensitivity and low hydraulic conductivity (Leibo, 1980; Ambrosini *et al*., 2006). Although, several experiments have been performed using different vitrification protocols with matured and immature bovine oocytes (Vajta *et al*., 1998; Men *et al*., 2002; Albarracin 2005; Magnusson *et al*., 2008; Morato *et al*., 2008; Vieira *et al*., 2008; Sripunya *et al*., 2010, Zhou *et al*., 2010), survival rates after warming have been low, with embryo production rates between 0 to 13% (Vajta *et al*., 1998; Dinnyes *et al*., 2000; Morato *et al*., 2008; Sripunya *et al*., 2010; Zhou *et al*., 2010).

One of the alternatives to enhance survival rates after vitrification may be the selection of more competent oocytes for cryopreservation using a different technique other than the subjective evaluation of the oocyte morphology with a stereomicroscope, as is done today for commercial *in vitro* embryo production (De Loos *et al*., 1992). The brilliant cresyl blue (BCB) is a supravital staining technique that has been used successfully to identify the most competent oocytes in several species such as caprine (Rodriguez-Gonzalez *et al*., 2002, 2003; Urdaneta *et al*., 2003), equine (Pereira *et al*., 2010), porcine (Wongsrikeao *et al*., 2006; Antosik *et al*., 2009; Ishizaki *et al*., 2009; Pawlak *et al*., 2011), bubaline (Manjunath *et al*., 2007; Heleil and Fayed, 2010), murine (Wu *et al*., 2007), and bovine (Pujol *et al*., 2004; Alm *et al*., 2005; Ghanem *et al*., 2007; Opiela *et al*., 2008; Torner *et al*., 2008; Hadi *et al*., 2010; Silva *et al*., 2013). The BCB technique estimates the activity of glucose-6-phosphate dehydrogenase (G6PDH), an enzyme that is synthesized in growing oocytes, but with decreased activity when oocytes have finished their growing phase (Tian *et al*., 1998). The evaluation is relatively simple, because oocytes exposed to BCB show a blue coloration when the cytoplasm has low G6DPH levels, because they do not reduce BCB to a colorless compound. Thus, this stain is a tool that could be used to enhance the selection of oocytes by visual evaluation and provides a more selected group of competent oocytes that could be vitrified and/or matured *in vitro* for embryo production. Hadi *et al*. (2010) have shown that selection by BCB staining increased the survival rates of vitrified

[5]Corresponding author: gabrielbo@iracbiogen.com.ar
Phone: +54(0)3543-477214
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immature oocytes; however, they only evaluated nuclear maturation and did not make an attempt to produce embryos *in vitro*.

Another unresolved question in relation to oocyte cryopreservation has been the possibility of using mature or immature oocytes. Although several reports have shown that oocyte survival after cryopreservation for mature *vs*. immature oocytes are different (Hurtt *et al*., 2000; Men *et al*., 2002; Albarracin *et al*., 2005; Magnusson *et al*., 2008; Zhou *et al*., 2010), the results have been controversial and require further investigation.

Hyaluronan is a glycosaminoglycan that has shown to have an important role in cell proliferation, cell migration, cell differentiation and gene expression regulation (Toole, 2001) and has been used as replacement for bovine serum in embryo media (Palasz *et al*., 2006). Its use into bovine culture has been shown to increase *in vitro* embryo development rates and survival rates after cryopreservation (Stojkovic *et al*., 2002; Dattena *et al*., 2007; Palasz *et al*., 2006, 2008; Sheehan *et al*., 2007; Block *et al*., 2009). Furthermore, hyaluronan has been used as a biopolymer for slow-release administration of FSH due to its molecular weight and chemical characteristics (Tríbulo *et al*, 2011). In aqueous solutions, hyaluronan has a tendency to form a three-dimensional network (Kobayashi *et al*., 1994), retaining critical concentrations in a diminutive volume of fluid surrounding the oocyte or embryo. Therefore, it could be used as a less toxic and more stable extracellular cryoprotectant into the vitrification solution, that could increase the developmental rates of vitrified-thawed oocytes regardless of the stage. The aim of the present study was to evaluate the effect of BCB on oocyte selection previous to cryopreservation and the addition of a biopolymer such as hyaluronan to the vitrification solution on developmental competence of immature and mature oocytes after vitrification.

Materials and Methods

Experimental design and allocation of treatments

Except where otherwise indicated, all chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

The study involved the vitrification of immature (n = 496) and *in vitro* matured (n = 392) oocytes selected by BCB and vitrified in two different solutions as shown in Fig. 1. Immature COCs were selected by the BCB stain method (BCB + or BCB-) and then randomly subdivided to be matured or not before vitrification using two solutions (VS1 or VS2). A group of immature COCs (Control fresh group, n = 220) were maintained in holding medium (Vigro® Holding, Bioniche Animal Health, Belleville, ON, Canada) for 90 min, as those that were exposed to the BCB stain, and then directly matured fertilized and cultured; whereas another group of COCs were also maintained in holding medium for 90 min (Control vitrified, n = 200) and were vitrified using the VS1 solution and then thawed, matured, fertilized and cultured *in vitro*. The study was performed in 10 replicates and approximately 120 to 150 oocytes were processed in each replicate.

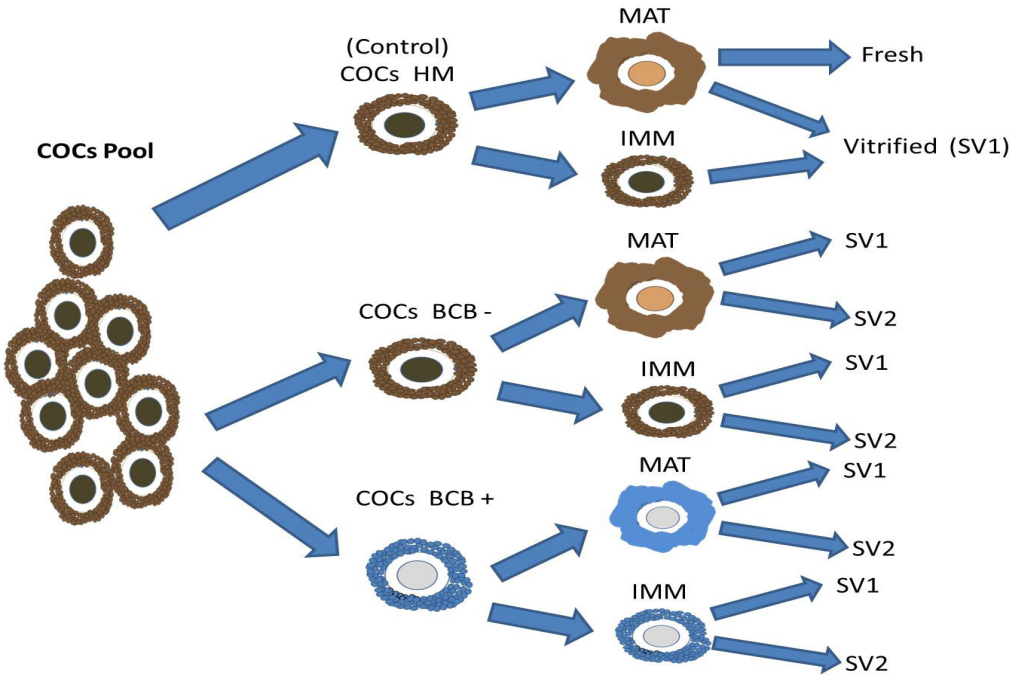

Figure 1. Experimental design. COCs: Cumulus-oocyte complexes; BCB+ or BCB-: Brillant cresyl blue positive or negative COCs; MAT: COCs matured *in vitro*; IMM: immature COCs; VS1 and VS2: vitrification solutions.

Oocyte source

Bovine ovaries from Angus cross-breed cows obtained from a local abattoir were transported to the laboratory within 2-4 h after slaughter, at 24-32°C, into Dulbecco's phosphate buffer saline. Cumulus-oocyte complexes (COCs) were aspirated from follicles 2-8 mm in diameter, washed in holding medium and classified according to morphological characteristics and only those with evenly granulated cytoplasm and surrounded by a compact and dense cumulus cell layer were used (De Loos *et al*., 1992).

Brilliant cresyl blue staining

The BCB staining was performed following the protocol indicated by Alm *et al*. (2005). Immediately after morphological selection, COCs were alocated in 100 µl drops of 26 µm BCB (Sigma, B-5388) in modified PBS containing 1.090 mg/ml glucose, 35.2 mg/ml (mol/l) pyruvate, 0.4% (w/v) BSA fraction V (Sigma A-9647), and incubated at 38.8°C, in a humidified atmosphere with 5% CO_2 for 90 min. After the incubation time, the oocytes were observed under stereomicroscope and classified according to BCB staining as: BCB + (oocytes with dark blue cytoplasm) or BCB- (oocytes with colorless cytoplasm). The control group were oocytes incubated for 90 min and placed in maturation media without any exposure to the stain.

Vitrification and warming

For vitrification, oocytes (*in vitro* matured or immature) were exposed to two different vitrification solutions. Oocytes vitrified in VS1 were first exposed to a solution containing 10% (v/v) ethylene glycol (EG, Sigma E-9129) in holding medium for 10 min for initial oocyte cryoprotectant saturation (Papis *et al*., 2000). Then, groups of 5-6 COCs were placed in 20% EG + 0.2M trehalose solution for 30 sec and subsequently in a 30% EG + 0.5M trehalose solution for 30 sec. Oocytes Vitrified in VS2 were first exposed to a 10% EG solution for 10 min, then 5-6 COCs were placed in a 20% EG + 0.2M trehalose solution for 30 sec, and finally into 30% EG + 0.5M trehalose + 0.1 mg/ml of hyaluronan for 30 sec. Oocytes exposed to the vitrification solution (1 µl) were then loaded into Fyberplugs™ and were vitrified using the Solid Surface Cryologic Vitrification Method (CVM, Cryologic Inc., Victoria, Australia) as previously described (Rodriguez-Villamil *et al*., 2013). Vitrified oocytes were stored in liquid nitrogen for at least one week. After that period embryos were thawed by placing the Fiberplugs™ directly into a 0.5M sucrose solution (in holding medium) warmed at 37°C for 5 min and then into a 0.25M sucrose solution for another 5 min and finally placed into TCM-199 culture medium.

In vitro *maturation*

Previously vitrified and non-vitrified COCs were matured as previously described (Rodriguez-Villamil *et al*, 2013). Briefly, COCs were matured in 500 µl drops in a 96-well culture plate (30-50 COCs in each well) and matured at 38.8°C in a moist atmosphere of 5% CO_2 in air for 24-26 h. The maturation medium consisted of TCM-199 supplemented with 10% (v/v) fetal bovine serum, 0.2 mM sodium pyruvate, 35 mg/ml of porcine FSH (Folltropin-V, Bioniche Animal Health) and antibiotics.

Fertilization and embryo culture

COCs were fertilized and cultured as previously described (Rodriguez-Villamil *et al*., 2013). For fertilization, each straw of frozen semen of an Angus bull was thawed for 1 min at 37°C, sperm were selected using Percoll discontinuous gradient (45 and 90%). In summary, the 90% Percoll solution (500 µl) was placed in a 2-ml Eppendorf tube, and 500 ul of 45% Percoll were smoothly layered on this. Frozen-thawed semen was layered on top of gradients and tubes were centrifuged for 15 min at 700 × g. The pellets were re-suspended in the same amount of sperm-TALP medium and centrifuged for 5 min at 700 × g. The supernatant was removed and the final concentration of the sperm pellet was adjusted with Fert-TALP to 1×10^6 sperm/ml for fertilization. COCs were fertilized in 500 µl droplets (30-50 COCs/droplet) covered with mineral oil into modified TALP fertilization medium. After 20 h of *in vitro* fertilization, the COCs were vortexed to remove the cumulus cells and excess sperm, washed once in culture medium and transferred into 500 µl drops of SOF culture medium under mineral oil in a controlled atmosphere (5% CO2, 5% O2, and 90% N2) at 38.8°C for 7 days.

Assessment of in vitro *development*

Cleavage rates of oocytes to the two-cell stage and development rates to the blastocyst stage were examined on days 2 and 7 after fertilization, respectively.

Statistical analysis

Cleavage and blastocyst rates were transformed by square root and then analyzed by ANOVA, considering maturation, BCB stain and vitrification solutions as main effects and their interactions. The Protected Least Significant Difference (LSD) test was used for subsequent multiple comparisons when ANOVA revealed statistically significant differences ($P < 0.05$). All data were analyzed using the software Infostat (UNC, Argentina, 2010).

Results

The effects of BCB staining on developmental and cleavage rates are depicted in Table 1. Cleavage and development rates of vitrified oocytes were greater for those selected as BCB+ than those selected as BCB - ($P < 0.001$; Table 1). Furthermore, cleavage and developmental rates of oocytes in the Control vitrified group (vitrified without stain selection) were similar to those in the BCB- group, but they were also lower than those selected as BCB + ($P < 0.001$). Finally, developmental rates in the Control fresh group were greater than those in the three vitrification groups. No interactions were detected between BCB staining and oocyte stage of maturity and vitrification solution.

The effect of oocyte stage of maturation on developmental and cleavage rates is depicted in Table 2. Although cleavage rates were similar for immature and *in vitro* matured oocytes after thawing, blastocyst rates were significantly higher in immature than in *in vitro* matured vitrified oocytes ($P < 0.001$; Table 2).

The effects of vitrification solution on developmental and cleavage rates are depicted in Table 3. The addition of hyaluronan to the vitrification solution (VS2) had no significant effect on cleavage and blastocyst rates. However, developmental rates of both vitrification groups were significantly lower than those from non-vitrified oocytes (Control fresh group; $P < 0.001$).

Table 1. Cleavage and blastocyst rates (means ± SEM) of vitrified-thawed bovine oocytes selected or not by brilliant cresyl blue (BCB).

BCB Selection	No. Oocytes	Cleavage rate %	Blastocyst rate %
BCB +	442	46.1 ± 2.4^{b}	5.0 ± 0.63^{b}
BCB -	446	32.1 ± 2.0^{c}	1.0 ± 0.4^{c}
Control vitrified	200	40.0 ± 4.0^{c}	2.0 ± 1.1^{c}
Control fresh (not vitrified)	220	62.9 ± 2.4^{a}	32.0 ± 2.4^{a}

[a,b,c] Rates in the same column with different superscripts differ significantly, $P < 0.05$. BCB +: Oocytes selected as brilliant cresyl blue positive; BCB -: Oocytes selected as Brilliant cresyl blue negative; Control vitrified: oocytes vitrified without BCB selection; Control fresh: oocytes without any treatment.

Table 2. Cleavage and blastocyst rates (means ± SEM) of different type of bovine oocyte (immature or *in vitro* matured) after vitrification.

Type of oocyte	No. Oocytes	Cleavage rate %	Blastocyst rate %
Immature	496	44.8 ± 1.9^{b}	4.0 ± 0.6^{b}
Mature	392	38.3 ± 2.8^{b}	2.5 ± 0.6^{c}
Control fresh (not vitrified)	220	62.9 ± 2.4^{a}	32.0 ± 2.4^{a}

[a,b,c]Rates in the same column with different superscripts differ significantly, $P < 0.05$

Table 3. Cleavage and blastocyst rates (means ± SEM) of bovine oocytes vitrified in two different vitrification solutions composed or not with hyaluronan.

Vitrification Solution	No. Oocytes	Cleavage rate %	Blastocyst rate %
VS1	445	39.3 ± 2.6^{b}	2.7 ± 0.6^{b}
VS2	443	43.7 ± 2.3^{b}	3.8 ± 0.7^{b}
Control fresh (not vitrified)	220	62.9 ± 2.4^{a}	32.0 ± 2.4^{a}

[a,b]Rates in the same column with different superscripts differ significantly, $P < 0.05$. VS1: oocytes vitrified in 10% (v/v) ethylene glycol for 10 min, followed by 20% EG + 0.2M trehalose for 30 sec and subsequently in a 30% EG + 0.5M trehalose for 30 sec. VS2: oocytes vitrified in 10% EG solution for 10 min, followed by 20% EG + 0.2M trehalose for 30 sec, and subsequently in 30% EG + 0.5M trehalose + 0.1 mg/ml of hyaluronan for 30 sec. Control fresh: oocytes without any treatment.

Discussion

Although blastocyst production was in general very low with vitrified oocytes, the results of the present study have shown that selection of oocytes using BCB may be a viable alternative to improve survival rates of vitrified oocytes.

The proportion of oocytes that are now utilized for *in vitro* production and cryopreservation in the commercial bovine *in vitro* embryo production is very low (Hwang and Hochi, 2014). To obtain successful results it is important to have oocytes with good quality and competence (Sirard *et al*., 2006), because only a percentage of the recovered oocytes may survive the vitrification process.

Most *in vitro* production laboratories used the morphological evaluation to select oocytes of "good" quality, demonstrating that there is a correlation between the integrity of cytoplasm and COCs with oocyte competence and consequently blastocyst production rates (Hawk and Wall, 1994; Fair, 2003; Merton *et al*., 2003; Pontes *et al*., 2010). Nevertheless, BCB staining has been shown to enhance these rates when immature oocytes were selected using G6DPH activity (Rodriguez-Gonzalez *et al*., 2003; Pujol *et al*., 2004; Alm *et al*., 2005; Wu *et al*., 2007; Opiela *et al*., 2008; Pereira *et al*., 2010; Silva *et al*., 2013). In the present study, BCB positive oocytes had higher developmental rates after thawing than BCB negative oocytes. Furthermore, BCB positive oocytes had greater developmental rates than the non BCB selected group of oocytes in the vitrification control group. These results also confirm those of other studies which demonstrated that BCB have the capacity to identify oocytes with higher developmental competence (Rodríguez-Gonzalez *et al*., 2003; Alm *et al*., 2005; Wu *et al*., 2007; Manjunath *et al*., 2007; Ishizaki *et al*., 2009; Heleil and Fayed, 2010; Silva *et al*., 2013). Moreover, the higher blastocyst rates obtained from the oocytes selected as BCB+ after warming in comparison with the other groups, confirm the observation of Hadi *et al*. (2010), in which more BCB+ oocytes developed to the MII stage than oocytes not selected by BCB prior to vitrification.

In this experiment, we obtain an enhancement of our embryo production. With the BCB+ group, we had 3% more blastocyst than the control group without any selection, that in terms of numbers were 20 more embryos for the oocytes selected as BCB + (n = 25) than the selected as BCB - (n = 5). This would represent a significant commercial advantage, with a higher number of pregnancies and less waste material on oocytes that could not be suitable for IVF or vitrification. This chemical selection demonstrated to be more efficient and accurate than the subjective visual assessment of oocyte quality.

Regarding vitrification, different survival rates after thawing have been reported between mature and immature oocytes. Some authors suggested that immature oocytes are more cryosensitive due to their low membrane stability and the susceptibility of their cytoskeleton (Men *et al*., 2002), but others observed increased chromosomal abnormalities, alterations of the distributions of cortical granules and alterations in the meiotic spindle in mature oocytes (Hyttel *et al*., 2000). However, all these reports (Hyttel *et al*., 2000; Men *et al*., 2002) are variable and even contradictory, due to the differences between protocols and laboratory procedures. Our results indicated that matured oocytes are more susceptible to vitrification damage because they presented lower developmental rates after thawing than immature oocytes.

Our results are in agreement with those reported by Le Gal and Massip (1999), Magnusson *et al*. (2008) and Zhou *et al*. (2010). The lower survival rates in *in vitro* matured oocytes were probably due to the expansion of cumulus cells that result in an increased area and volume, and also permeability changes that made them more sensitive to cryopreservation (Agca *et al*., 1998; Wang *et al*., 2010).

In relation to vitrification solutions, cleavage and blastocyst rates of oocytes vitrified with the addition of hyaluronan in the VS2 solution were only numerically (not significantly) higher than those from oocytes vitrified in solutions without hyaluronan (VS1). Previous studies reported an improved embryo cryosurvival after thawing when hyaluronan was added to the culture media (Stockjovic *et al*., 2002; Palasz *et al*., 2008, 2009; Block *et al*., 2009) or during a dehydration process previous to cryopreservation (Sheehan *et al*., 2007). Therefore, the use of hyaluronan may be more effective when used in a pre-incubation process prior to cryopreservation rather than adding hyaluronan to the vitrification solution itself, as was done in the present study. According to our results, Palasz *et al*. (2000) demonstrated the efficacy of sodium hyaluronan (SH) in the freezing solutions as a replacement for other macromolecules as BSA or embryo transfer surfactant (ETS), but no improvements were detected compared to BSA or surfactant.

In conclusion, the selection of oocytes by BCB and the use of immature oocytes increased the development rates of vitrified-warmed oocytes. However, the addition of hyaluronan in the vitrification solutions did not enhance oocyte survival rate after warming. Finally, bovine oocyte cryopreservation still requires further improvements before it can be successfully used in the field.

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