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Effect of two Different Permitting and Combination of Cryoprotectants on Cattle Oocytes Maturation Rate Following Brilliant Cresyl Blue Exposure

[1,3]Masindi Lottus Mphaphathi, [3]Hester Adri O'Neill and [1,2]Tshimangadzo Lucky Nedambale

[1]Agricultural Research Council, Animal Production, Germplasm Conservation and Reproductive Biotechnologies, Private Bag X 2, Irene, 0062, South Africa
[2]Tshwane University of Technology, Department of Animal Sciences, Faculty of Science, Private Bag X 680, Pretoria, 0001, South Africa
[3]University of the Free State, Department of Animal, Wildlife and Grassland Sciences, P.O. Box 339, Bloemfontein, 9300, South Africa

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Corresponding Author:
Masindi Lottus Mphaphathi
Agricultural Research Council, Animal Production, Germplasm Conservation, and Reproductive Biotechnologies, Private Bag X 2, Irene, 0062, South Africa
Email: masindim@arc.agric.za

Abstract: The objectives of this study were to elucidate the toxicity of Dimethyl sulfoxide (DMSO), Ethylene glycol (EG), and the combination of both permitting cryoprotectants (CPAs) to cattle oocytes and the effectiveness of Brilliant cresyl blue (BCB) on immature oocytes pre-selection. Cattle ovaries were collected from the local slaughterhouse and transported within 2 hours of slaughter. The oocytes were exposed to 26 mM BCB solution. The other oocytes were not exposed to BCB solution or CPA. Oocytes were classified as BCB positive (+) or BCB negative (−). Oocytes were either exposed to DMSO, EG, or DMSO + EG at different CPA concentrations as follows: toxicity test 1 (TT1) was 0, 5, 10, 15, and 20%, followed by exposure to TT2 as follows: 10, 20, 30 and 40%. The oocytes were then *in vitro* matured as per treatment groups. After maturation, oocyte polar body extrusion was evaluated. Treatment means were compared using the Fisher-protected t-test least significant difference. The DMSO treatment group: The oocytes with polar body extrusion were 55.1% (positive control, no BCB and no CPA exposure), 55.0% (control, CPA exposure no BCB), 22.7% [BCB− with CPA toxicity test (DMSO 5 + 10%)], 21.8% [BCB− with CPA toxicity test (DMSO 10 + 20%)] and 7.3% [BCB− with CPA toxicity test (DMSO 20 + 40%)]. The BCB + groups (DMSO 5 + 10 and DMSO 10 + 20%) had more oocytes with polar body extrusion (67.0 and 66.9%, respectively; $P>0.05$). The EG treatment group: There was a drastic decline of oocytes with polar body extrusion as EG CPA concentration increased ($P<0.05$). The BCB+ groups (EG 5 + 10 and EG 10 + 20%) had significantly more oocytes with polar body extrusion (68.9 and 51.9%). The DMSO + EG treatment group: There was a decline in oocytes polar body extrusion recorded, as the DMSO + EG CPAs concentrations were increased ($P<0.05$). The BCB+ groups (DMSO 5 + EG 5% and DMSO 10 + EG 10%) recorded numerically highest number of oocytes with polar body extrusion (55.1 and 55.8%) as compared to positive control (51.1%), respectively ($P>0.05$). In conclusion, a decline in oocyte polar body extrusion was recorded, as the DMSO, EG, and DMSO + EG concentrations were increased. The BCB can be used to identify developmentally competent oocytes.

Keywords: Ethylene Glycol, Cattle Oocytes, Toxicity, Cryoprotectants

Introduction

The selection of quality oocytes among a heterogeneous pool is mostly done subjectively. The use of Brilliant cresyl blue (BCB) stain for oocyte selection before the cryoprotectant (CPA) toxicity test on cattle oocytes might be a useful tool. To date, toxicity trials have recorded unpredictable outcomes with no consensus on the least, suitable concentration and most toxic CPA.

The BCB stain is known to be a non-invasive methodology that allows the selection of oocytes with larger diameters amongst a heterogeneous pool (Catala *et al*., 2011). Despite the overall positive results obtained with BCB selection for oocytes, the effectiveness of this test is still controversial, to date (Piras *et al*., 2020). The BCB test determines the intracellular activity of glucose-6-phosphate dehydrogenase (G6PDH), a pentose phosphate pathway enzyme that gradually decreases its activity as oocytes reach their growth phase. The BCB dye can be reduced by G6PDH activity, therefore oocytes that have reached their growth phase cannot reduce BCB to a colorless compound and exhibit a blue-colored cytoplasm. However, growing oocytes are expected to have a high level of G6PDH activity and be able to reduce the blue compound, resulting in a colorless oocyte cytoplasm (Catala *et al*., 2011; Lee *et al*., 2020).

In vitro maturation (IVM) is probably the most critical part of the whole process of *in vitro* embryo production (Ferré *et al*., 2020; Leal *et al*., 2020). Ruminant oocytes are usually matured at 39°C under 5% CO_2 in a humidified atmosphere. The optimal maturation time (approximately 90% of the oocytes at the metaphase II stage) is 22-24 hours. The developmental potential of *in vivo* matured oocytes is known to be greater than that of IVM oocytes. It has been demonstrated that the *in vitro* development of *in vivo* matured oocytes is twice as high as that of IVM oocytes because important molecular changes occur during *in vivo* capacitation (Hendriksen *et al*., 2000; Dieleman *et al*., 2002; Kirillova *et al*., 2021).

Cryoprotectants (CPAs) are compounds used in cryoprotective solutions to achieve cellular dehydration and to avoid intracellular ice crystal formation upon freezing (Prentice and Anzar, 2010). The toxicity of permitting CPA has been a limiting step for the use of high CPA concentrations. This is critical to the structural integrity and the survivability of cells post cryopreservation (Whaley *et al*., 2021) and for the improvement of cryopreservation protocols for mammalian cells (Pegg, 2007; Fahy *et al*., 1987). The other strategy to deal with this issue is to use less toxic but reasonably efficient CPA (Szurek and Eroglu, 2011).

A better understanding of the mechanisms of CPA toxicity to know how to reduce its toxicity could be a breakthrough in the field of cryobiology (Best, 2015). The exposure of oocytes to very high concentrations of CPAs is known to damage oocytes/cells because of both osmotic and toxic effects (Cai *et al*., 2005; Al-Azawi *et al*., 2013). Dimethyl sulfoxide (DMSO), Ethylene glycol (EG), Glycerol, Propylene glycol, etc, are being used as permitting CPAs for oocytes (Pedro *et al*., 2005; Mahmoud and El-Sokary, 2013; Best, 2015). Evaluation of permitting CPA solution toxicity is therefore very important prior-vitrification/cryopreservation of oocytes.

We hypothesized that DMSO and BCB+ oocytes groups will have higher percentages of oocytes with polar body extrusion following IVM. Therefore, the current study aimed to elucidate the toxicity of DMSO, EG, and the combination of DMSO + EG permitting CPA to cattle oocytes and the effectiveness of BCB stain on immature oocytes pre-selection.

Materials and Methods

Chemicals and Reagents

Chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise stated. All chemicals were of analytical grade.

Cattle Ovaries Collection and Oocytes Retrieval

Heterogeneous ovaries of unknown reproductive status cattle were collected from the local slaughterhouse and transported to the laboratory in a normal saline solution (0.9%, Adcock Ingram Critical Care, Pty, Ltd, South Africa) in a thermos-flask at 37°C, within 2 hours of slaughter. The aspiration method was used to retrieve the oocytes from the ovaries. During the aspiration method, cumulus-oocyte complexes were aspirated from 2 to 7 mm diameter follicles. The disposable 18-gauge needle (Neojet, UK) was attached to the 10 mL syringe (Neomedic, UK). The follicular fluid containing oocytes was placed into a 50 mL plastic tube (Falcon) during the aspiration process, until further evaluation.

Oocytes Selection by Brilliant Cresyl Blue Stain

Immediately after collection, oocytes were washed three times in a warm solution of modified phosphate-buffered saline (mPBS) and exposed to 26 mm of BCB stain in mPBS media for 90 minutes at 38.5°C under an atmosphere of 5% CO_2 air with 100% humidity. The exposed cumulus-oocyte complex was then washed two times in mPBS followed by three times wash in Medium 199 (M199) + 10% fetal bovine serum (FBS) and examined under the microscope. Oocytes were either classified as BCB positive (+) with a varying degree of blue cytoplasm or BCB negative (−) with no blue cytoplasm or hypothetically growing oocytes and recorded (Ogata *et al*., 2016).

Immature Oocytes Toxicity Test

The control oocytes were not exposed to BCB solution and CPA [No BCB and No CPA exposure (positive control)]. While the other oocytes were exposed to CPA but not the BCB treatment group. The CPAs concentration for DMSO, EG, and DMSO + EG were: toxicity test 1 (TT1) was as follows 0, 5, 10, 15, and 20% and then followed by exposure to TT2 as follows 10, 20, 30, and 40% (stepwise

increased CPAs). The oocytes were then washed three times in M199 + 10% FBS. The duration for oocytes into TT1 was 3 minutes followed by TT2 for 30 seconds.

In-vitro Maturation of Oocytes and Polar Body Evaluation

Oocytes were then subjected to IVM (M199 medium) with 10% FBS, luteinizing hormone, follicle-stimulating hormone, and estradiol hormone as per treatment groups for 22 hours at 38.5°C under an atmosphere of 5% CO_2. After maturation, oocytes were removed from the maturation medium and denuded of granulosa cells by vortexing and also by gentle pipetting with a hand pipette. The oocyte's polar body extrusion was evaluated with the aid of the Oosight Imaging System (Hamilton Thorne) connected to an inverted research microscope (at 20 x magnification) and recorded.

Statistical Analysis

Data were analyzed using the statistical program GenStat at a significance level (P<0.05). Analysis of variance was used to establish differences in the effect of BCB stain on oocytes selection and toxicity of oocytes into different CPAs on oocytes polar body extrusion. Treatment means were separated using the Fisher's protected t-test least significant difference. The data were presented as mean ± standard deviation.

Results

The Effect of Different Concentrations of Dimethyl Sulfoxide Cryoprotectant on Oocytes Maturation Rate Following Brilliant Cresyl Blue Exposure

The oocytes with polar body extrusion was 55.1% (positive control, no BCB and no CPA exposure), 55.0% (control, CPA exposure no BCB), 22.7% [BCB− with CPA toxicity test (DMSO 5 + 10%)], 21.8% [BCB− with CPA toxicity test (DMSO 10 + 20%)] and 7.3% [(BCB− with CPA toxicity test (DMSO 20 + 40%)] (Table 1). The BCB+ groups (DMSO 5 + 10% and DMSO 10 + 20%) had more oocytes with polar body extrusion (67.0% and 66.9%) compared with the positive control (55.1%), respectively (P>0.05). All the treatments groups of oocytes exposed into DMSO 20 + 40% toxicity test had the lowest polar body extrusion (ranged from 7.7 to 13.1%) recorded as compared to lower CPA concentrations (P<0.05). Furthermore, all the BCB− treatments groups had the lowest oocytes polar body extrusion, irrespective of DMSO concentration (P>0.05).

The Effect of Different Concentrations of Ethylene Glycol Cryoprotectant on Oocytes Maturation Rate Following Brilliant Cresyl Blue Exposure

There was a drastic decline of oocytes with polar body extrusion as CPA concentration increased (P<0.05; Table 2): 40.5% (positive control, no BCB and no CPA exposure), 33.3% [control, CPA exposure (EG 5 + 10%)], 23.1% [BCB− with CPA toxicity test (EG 5 + 10%)], 12.5% [(BCB− with CPA toxicity test (EG 10 + 20%)] and 4.9% [BCB− with CPA toxicity test (EG 15 + 30%)]. The BCB+ groups (EG 5 + 10% and EG 10 + 20%) had significantly more oocytes with polar body extrusion (68.9 and 51.9%) as compared with the positive control (40.5%), respectively (P<0.05).

Table 1: Effect of different concentrations of Dimethyl sulfoxide cryoprotectant on cattle oocytes maturation rate following Brilliant cresyl blue exposure.

Treatment	DMSO CPA (%)	Total oocytes IVM	Polar body status (%)
Positive control (No BCB and No CPA exposure)	0	197	55.1±8.8^{a,b}
Control, CPA exposure (no BCB)	5+10	37	55.0±4.0^{a,b}
	10+20	50	52.6±4.6^{a,b}
	15+30	43	40.8±11.0^{b}
	20+40	58	16.2±6.5^{c}
BCB+	5+10	20	67.0±14.0^{a}
	10+20	22	66.9±6.3^{a}
	15+30	32	48.5±2.3^{b}
	20+40	42	13.1±6.1^{c}
BCB−	5+10	21	22.7±20.2^{c}
	10+20	38	21.8±6.1^{c}
	15+30	33	10.6±9.4^{c}
	20 + 40	33	7.3±8.8^{c}

$^{a-c}$Values with different superscript within a column are significantly different (P<0.05). Dimethyl sulfoxide (DMSO), *In vitro* maturation (IVM), Cryoprotectant (CPA) and Brilliant cresyl blue (BCB).

Table 2: Effect of different concentration of Ethylene glycol cryoprotectant on cattle oocytes maturation rate following Brilliant cresyl blue exposure

Treatment	EG CPA (%)	Total oocytes IVM	Polar body status (%)
Positive control (No BCB and No CPA exposure)	0	450	40.5±17.3^{b,c}
Control, CPA exposure (no BCB)	5 + 10	99	33.3±112.0^{c,d}
	10 + 20	135	30.2±16.2^{c-e}
	15 + 30	115	24.4±6.5^{c-e}
	20 + 40	71	15.8±14.2^{d-g}
BCB+	5 + 10	122	68.9±12.7^{a}
	10 + 20	60	51.9±19.0^{a,b}
	15 + 30	94	27.1±27.7^{c,d}
	20 + 40	48	11±7.2^{e-g}
BCB−	5 + 10	112	23.1±6.5^{c-f}
	10 + 20	115	12.5±13.2^{e-g}
	15 + 30	99	4.9±4.1^{f,g}
	20 + 40	56	0.0±0.0^{g}

$^{a-g}$Values with different superscript within a column are significantly different (P<0.05). Ethylene glycol (EG), *In vitro* maturation (IVM), Cryoprotectant (CPA) and Brilliant cresyl blue (BCB).

Table 3: Effect of different concentration of Dimethyl sulfoxide + Ethylene glycol cryoprotectants on cattle oocytes maturation rate following Brilliant cresyl blue exposure

Treatment	DMSO + EG CPA (%)	Total oocytes IVM	Polar body status (%)
Positive control (No BCB & No CPA exposure)	0	118	51.1±6.2^{a}
Control, CPA exposure (no BCB)	5 + 5	129	48.7±3.1^{a,b}
	10 + 10	116	50.4±3.1^{a}
	15 + 15	122	25.0±10.9^{c}
	20 + 20	119	15.5±4.9^{d,e}
BCB+	5 + 5	111	55.8±5.4^{a}
	10 + 10	92	55.1±5.7^{a}
	15 + 15	102	42.0±7.7^{b}
	20 + 20	112	20.9±4.7^{c,d}
BCB−	5 + 5	112	8.2±8.8^{e}
	10 + 10	109	19.4±9.4^{c,d}
	15 + 15	93	8.2±8.4^{e}
	20 + 20	90	7.8±7.2^{e}

$^{a-e}$Values with different superscript within a column are significantly different (P<0.05). Dimethyl sulfoxide (DMSO), Ethylene glycol (EG), *In vitro* maturation (IVM), Cryoprotectant (CPA) and Brilliant cresyl blue (BCB).

The Effect of Different Concentration of Dimethyl Sulfoxide + Ethylene Glycol Cryoprotectants on Oocytes Maturation Rate Following Brilliant Cresyl Blue Exposure

There was a decline on oocytes polar body extrusion recorded, as the DMSO + EG CPAs concentrations were increased (P<0.05; Table 3): 51.1% (positive control, no BCB and no CPA exposure), 48.7% (control, CPA exposure- no BCB), 8.2% [BCB− with CPA toxicity test (DMSO 5 + EG 5%)], 19.4% [BCB− with CPA toxicity test (DMSO 10 + EG 10%)] and 7.8% [BCB− with CPA toxicity test (DMSO 20 + EG 20%)]. The BCB+ groups (DMSO 5 + EG 5% and DMSO 10 + EG 10%) recorded numerically highest number of oocytes with polar body extrusion (55.1% and 55.8%) as compared to positive control (51.1%), respectively (P>0.05). The treatments groups of oocytes exposed into DMSO 20 + EG 20% toxicity test had recorded the lowest polar body extrusion (ranged from 7.8 to 20.9%) as compared to lower CPA concentrations (P<0.05).

Discussion

This study elucidates the toxicity of DMSO, EG, and the combination of DMSO + EG permitting CPA to cattle oocytes and the effectiveness of BCB stain on immature oocytes pre-selection. The findings of the present study indicated that the increase of DMSO or EG or a combination of both drastically decreases the oocyte's maturation rate (polar body extrusion). The oocytes maturation rate ranged from 40.5 to 55.1% on the oocytes that were not exposed to CPA and BCB (control group). In the oocytes that were exposed to CPA's toxicity (no BCB exposure), their maturation rate ranged from 15.5 to 55.0%. The BCB (+ and −) oocytes (selected) showed some differences in oocytes' polar body extrusion percentages following IVM in different CPA/s percentages. The oocyte maturation rate for BCB+ groups (DMSO: 10, 20, 30, and 40%) ranged from 13.1 to 67.0% and was the lowest as compared to the BCB–

groups and ranged from 7.3 to 22.7%. Many of the differences in the results of oocyte CPA toxicity research among the labs arise because of different experimental conditions, such as temperature, CPA concentration, duration of exposure to CPA, and choice of CPA (Fahy *et al*., 1990; Wusteman *et al*., 2002).

In the current study, mature oocytes (BCB+) had high affinity for BCB stain than immature oocytes. This was attributed to the G6PDH enzyme that was synthesized in oocytes during oogenesis. However, this enzyme was inactive in oocytes that finished their growth phase. When oocytes completed their growth phase, G6PDH activity was too small to reduce the staining so had blue coloration of cytoplasm (BCB+) (grown) oocytes. While the growing oocytes remain colorless (BCB–). Reduced G6PDH activity could be used as an indicator for induction of cytoplasmic maturation and to obtain the acceptable maturation rates on oocytes that were exposed to toxicity tests for CPAs.

The molecular weight for DMSO (78.13 g/mol) and EG (62.07 g/mol) are different in the rate of permitting the oocytes *in vitro*. The negative impact on EG oocytes groups (without BCB exposure) revealed a lower oocyte with polar body extrusion as compared to DMSO and DMSO + EG groups. The oocyte maturation rate for EG groups (without BCB exposure) ranged from 15.8 to 33.3%, for BCB+ groups ranged from 11.7 to 68.9% and the lowest was on the BCB– groups. Different penetrating CPAs such as DMSO, EG, Glycerol, Propylene glycol, and Acetamide have been used. Exposing cattle oocytes to a low concentration of permeating cryoprotectant (3 or 4% by volume) before exposure to the vitrification solution may be more beneficial than using a higher concentration for the first step, as is usually done in some labs (Mullena and Fahy, 2012), but reasonably efficient CPAs (Szurek and Eroglu, 2011). The DMSO as a dipolar aprotic solvent (solvent that cannot donate a hydrogen bond) has an array of properties that allow its participation in numerous chemical reactions (Awan *et al*., 2020).

The oocytes tend to possess some characteristics that are an obstacle to CPA equilibrium between intra and extracellular environments. The oocyte is a large single cell with a small surface/volume ratio and is surrounded by several layers of intact cumulus cells, which compromises the CPA adequate entry into the cell (Massip, 2003). Factors that provide CPA passage velocity through the cell membrane and entry into the ooplasm are vital for oocyte pre-vitrification or freezing. These problems notwithstanding to date, an appropriate phased composition of CPA mitigates the toxic and osmotic consequences of highly concentrated CPAs (Cocchia *et al*., 2010). Prentice *et al.* (2011) reported that the CPA toxicity of DMSO and EG might be reduced by using combinations of more than one CPA and a stepwise exposure to cells. Thus, a mixture of CPAs might be beneficial and decreases individual-specific toxicity. The maturation rate for DMSO + EG (without BCB exposure) ranged from 15.5 to 50.4, selected oocytes (BCB+) their maturation rate ranged from 20.9 to 55.8%. The positive interaction of CPAs can be explained by the fact that the less toxic EG tends to leave the cells rapidly and the addition of the DMSO tend to allow the presence of CPA within the cells in a more efficient manner (Elliott *et al.*, 2017). The previous studies suggested that the toxicity of penetrating CPAs increases as the CPA concentration increases too, with higher exposure temperature and longer exposure time (Szurek and Eroglu, 2011). In the present study, it was evident that an increase in CPA reduces the oocyte's maturation success *in vitro*. Exposure to permitting CPAs can independently induce depolymerization/disruption of the oocyte cytoskeleton such as microfilaments and meiotic spindle microtubules, possibly leading to dispersion and inappropriate segregation of chromosomes, failure in oocytes polar body formation, and thus chromosomal abnormalities (Pickering and Johnson, 1987).

Conclusion

In conclusion, higher DMSO, EG, and DMSO + EG (combination of both) CPAs concentrations compromise oocyte polar body extrusion following IVM. It is recommended that BCB be used for the selection of suitable oocytes before the CPA toxicity test because of its ability to stain larger and more competent oocytes from cattle.

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Author's Contributions

Masindi Lottus Mphaphathi: Conception, design, data collection, analysis, interpretation, drafting, and reviewing of the article.

Hester O'Neill and Tshimangadzo L. Nedambale: Conception, design, reviewing of the article.

Ethics

Experimental cattle ovaries (oocytes) were cared for, according to the guidelines of the Agricultural Research Council (ARC)-Animal Production ethics committee (Ref: APIEC18/02).

References

Al-Azawi, T., Tavukcuoglu, S., Khaki, A. A., & Al Hasani, S. (2013). Cryopreservation of human oocytes, zygotes, embryos and blastocysts: A comparison study between slow freezing and ultra rapid (vitrification) methods. Middle East Fertility Society Journal, 18(4), 223-232. doi.org/10.1016/j.mefs.2012.10.008

Awan, M., Buriak, I., Fleck, R., Fuller, B., Goltsev, A., Kerby, J., & Stacey, G. N. (2020). Dimethyl sulfoxide: a central player since the dawn of cryobiology, is efficacy balanced by toxicity? Regenerative medicine, 15(3), 1463-1491. doi.org/10.2217/rme-2019-0145

Best, B. P. (2015). Cryoprotectant toxicity: Facts, issues, and questions. Rejuvenation research, 18(5), 422-436. doi.org/10.1089/rej.2014.1656

Cai, X. Y., Chen, G. A., Lian, Y., Zheng, X. Y., & Peng, H. M. (2005). Cryoloop vitrification of rabbit oocytes. Human Reproduction, 20(7), 1969-1974. doi.org/10.1093/humrep/deh805

Catala, M. G., Izquierdo, D., Uzbekova, S., Morató, R., Roura, M., Romaguera, R., & Paramio, M. T. (2011). Brilliant cresyl blue stain selects largest oocytes with highest mitochondrial activity, maturation-promoting factor activity and embryo developmental competence in prepubertal sheep. Reproduction, 142(4), 517. doi.org/10.1530/REP-10-0528

Cocchia, N., Ciani, F., Russo, M., El Rass, R., Rosapane, I., Avallone, L., & Lorizio, R. (2010). Immature cat oocyte vitrification in open pulled straws (OPSs) using a cryoprotectant mixture. Cryobiology, 60(2), 229-234. doi.org/10.1016/j.cryobiol.2010.01.003

Dieleman, S. J., Hendriksen, P. J. M., Viuff, D., Thomsen, P. D., Hyttel, P., Knijn, H. M., ... & Vos, P. L. A. M. (2002). Effects of in vivo prematuration and in vivo final maturation on developmental capacity and quality of pre-implantation embryos. Theriogenology, 57(1), 5-20. doi.org/10.1016/s0093-691x(01)00655-0

Elliott, G. D., Wang, S., & Fuller, B. J. (2017). Cryoprotectants: A review of the actions and applications of cryoprotective solutes that modulate cell recovery from ultra-low temperatures. Cryobiology, 76, 74-91. doi.org/10.1016/j.cryobiol.2017.04.004

Fahy, G. M., Levy, D. I., & Ali, S. E. (1987). Some emerging principles underlying the physical properties, biological actions, and utility of vitrification solutions. Cryobiology, 24(3), 196-213. doi.org/10.1016/0011-2240(87)90023-x

Fahy, G. M., Lilley, T. H., Linsdell, H., Douglas, M. S. J., & Meryman, H. T. (1990). Cryoprotectant toxicity and cryoprotectant toxicity reduction: In search of molecular mechanisms. Cryobiology, 27(3), 247-268. doi.org/10.1089/rej.2014.1656

Ferré, L. B., Kjelland, M. E., Strøbech, L. B., Hyttel, P., Mermillod, P., & Ross, P. J. (2020). Recent advances in bovine *in vitro* embryo production: reproductive biotechnology history and methods. Animal, 14(5), 991-1004. doi.org/10.1017/S1751731119002775

Kirillova, A., Smitz, J. E., Sukhikh, G. T., & Mazunin, I. (2021). The role of mitochondria in oocyte maturation. Cells, 10(9), 2484. doi.org/10.3390/cells10092484

Leal, G. R., Graciosa, M. A. G., Monteiro, C. A. S., Pasolini, R., dos Reis Camargo, A. J., Oliveira, C. S., ... & Serapião, R. V. (2020). The SPOM-adapted IVM system improves *in vitro* production of bovine embryos. Theriogenology, 158, 277-282. doi.org/10.1016/j.theriogenology.2020.09.024

Lee, S., Kang, H. G., Jeong, P. S., Nanjidsuren, T., Song, B. S., Jin, Y. B., & Sim, B. W. (2020). Effect of oocyte quality assessed by Brilliant cresyl blue (BCB) staining on cumulus cell expansion and sonic hedgehog signaling in porcine during *in vitro* maturation. International Journal of Molecular Sciences, 21(12), 4423. doi.org/10.3390/ijms21124423

Mahmoud, K. G. H. M., & El-Sokary, M. M. M. (2013). Improvement of the efficacy of buffalo oocytes vitrification. Glob Vet, 11(4), 420-31. doi.org/10.5829/idosi.gv.2013.11.4.7645

Massip, A. (2003). Cryopreservation of bovine oocytes: current status and recent developments. Reproduction Nutrition Development, 43(4), 325-330. doi.org/10.1051/rnd:2003024

Mullen, S. F., & Fahy, G. M. (2012). A chronologic review of mature oocyte vitrification research in cattle, pigs, and sheep. Theriogenology, 78(8), 1709-1719. doi.org/10.1016/j.theriogenology.2012.06.008

Ogata, Y., Yu, G. M., Hidaka, T., Matzushige, T., & Maeda, T. (2016). Effective embryo production from Holstein cows treated with gonadotropin-releasing hormone during early lactation. Theriogenology, 86(6), 1421-1426. doi.org/10.1016/j.theriogenology2016.04.087

Pedro, P. B., Yokoyama, E., Zhu, S. E., Yoshida, N., Valdez Jr, D. M., Tanaka, M., & Kasai, M. (2005). Permeability of mouse oocytes and embryos at various developmental stages to five cryoprotectants. Journal of Reproduction and Development, 51(2), 235-246. doi.org/10.1262/jrd.16079

Pegg, D. E. (2007). Principles of cryopreservation. Cryopreservation and freeze-drying protocols, 39-57. doi.org/10.1007/978-1-59745-362-2-3

Pickering, S. J., & Johnson, M. H. (1987). The influence of cooling on the organization of meiotic spindle of the mouse oocyte. Human Reproduction, 2(3), 207-216. doi.org/10.1093/oxfordjournals.humrep.a136516

Piras, A. R., Ariu, F., Zedda, M. T., Paramio, M. T., & Bogliolo, L. (2020). Selection of immature cat oocytes with Brilliant cresyl blue stain improves *in vitro* embryo production during non-breeding season. Animals, 10(9), 1496. doi.org/10.3390/ani10091496

Prentice, J. R., Singh, J., Dochi, O., & Anzar, M. (2011). Factors affecting nuclear maturation, cleavage and embryo development of vitrified bovine cumulus-oocyte complexes. Theriogenology, 75(4), 602-609. doi.org/10.1016/j.theriogenology.2010.09.027

Prentice, J.R. & Anzar, M. (2010). Cryopreservation of mammalian oocyte for conservation of animal genetics. Veterinary Medicine International, 1-11. doi.org/10.4061/2011/146405

Szurek, E. A., & Eroglu, A. (2011). Comparison and avoidance of toxicity of penetrating cryoprotectants. PloS one, 6(11), e27604.

Whaley, D., Damyar, K., Witek, R. P., Mendoza, A., Alexander, M., & Lakey, J. R. (2021). Cryopreservation: An overview of principles and cell-specific considerations. Cell Transplantation, 30, 0963689721999617. doi.org/10.1177/0963689721999617

Wusteman, M. C., Pegg, D. E., Robinson, M. P., Wang, L. H., & Fitch, P. (2002). Vitrification media: Toxicity, permeability, and dielectric properties. Cryobiology, 44(1), 24-37. doi.org/10.1016/S00 11-2240 (02)00002-0