Journal of Animal and Veterinary Advances 9 (21): 2710-2713, 2010
ISSN: 1680-5593

Selection of Immature Bovine Oocytes Using Brilliant Cresyl Blue Enhances Nuclear Maturity after Vitrification

[1]Hajarian Hadi, [1]H. Wahid, [1]Y. Rosnina, [2]M. Daliri, [1]M. Dashtizad, [3]H. Karamishabankareh, [1]A. Faizah, [4]M.I. Iswadi and [4]O. Abas Mazni
[1]Department of Clinical Sciences, Faculty of Veterinary Medicine,
University Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[2]Department of Animal Biotechnology,
National Institute of Genetic Engineering and Biotechnology, Tehran, Iran
[3]Department of Animal Science, Faculty of Agriculture, Razi University, Kermanshah, Iran
[4]Depertment of Reproductive Biotechnologies, Agro-Biotechnology Institute,
P.O. Box 341, 43400 Serdang, Selangor, Malaysia

Abstract: Beside cooling/warming rates and composition of vitrification solution, developmental stage of immature oocytes may also affect their vitrification outcome. The aim of the present study was to evaluate the selection effect of developmentally competent immature bovine oocytes by Brilliant Cresyl Blue (BCB) on maturity of oocytes after vitrification. Oocytes were obtained from slaughterhouse ovaries. Only oocytes with 4-5 layers of cumulus cells and homogenous cytoplasm were used. After exposure to BCB stain, immature oocytes were divided into colored (BCB+) and colorless (BCB-) cytoplasm groups. Immature oocytes were equilibrated in VS1 (7.5 Ethylene Glycol (EG)+7.5% DMSO) for 10-12 min and then exposed to VS2 (15% EG+ 15% DMSO+0.5M sucrose) for 1 min. Thereafter, oocytes were loaded on Cryotop and directly plunged into liquid nitrogen. After warming, oocytes were examined for presence of polar body and nuclear maturity. Higher number of oocytes in BCB+group extruded first polar body in comparison with other vitrified groups but not significantly ($p>0.05$). Compared to the BCB- oocytes, there was significantly lower percentage of degeneration for BCB+oocytes ($p<0.05$). Within vitrified groups, reaching to the MII stage was significantly higher in BCB+group (51.5%) compared with BCB and vitrified-control groups (27.9 and 40.3%, respectively). These results indicated that selection of potent immature bovine oocytes using brilliant cresyl blue improved the nuclear maturity of immature oocytes after vitrification. In addition, this selection can be a valuable tool to improve the vitrification outcome.

Key words: Vitrification, brilliant cresyl blue, bovine, immature oocytes, Malaysia, Iran

INTRODUCTION

Most of the published studies in vitrification of oocytes/embryos focus on basics of cryobiology such as increasing cooling/warming rates (Morato *et al*., 2008), vitrification solution ingredients (Yamada *et al*., 2007) and temperature of equilibration solution (Hadi *et al*., 2010). Although, the previously studies resulted in more favorable results, still the trend of vitrification of immature bovine oocytes is not satisfactory enough. Besides the importance of cryobiological factors, developmental stage of immature oocyte may play a significant role not only for *in vitro* Embryo Production (IVEP) system but also for the vitrification outcome. Almost as a routine part of IVEP system activities, immature oocytes are selected based on morphology of oocyte and its surrounding cells (Hawk and Wall, 1994; Hazeleger *et al*., 1995). It has been shown that a portion of morphologically selected oocytes are still in growing phase and not yet ready for maturation step (Rodriguez-Gonzalez *et al*., 2002; Alm *et al*., 2005).

The pool of immature oocytes can be separated effectively using Brilliant Cresyl Blue (BCB) stain test (Rodriguez-Gonzalez *et al*., 2002; Manjunatha *et al*., 2007). The BCB test is non-invasive and measures Glucose-6-phosphate Dehydrogenize (G6PDH) activity. G6PDH is a protein synthesized in growing immature oocytes and converts BCB stain to colorless. Conversely, grown oocyte cytoplasm which has lower levels of G6PDH remains blue in color (BCB+) because there is no reduction for BCB to a colorless compound (Bhojwani *et al*., 2007; Ishizaki *et al*., 2009). Based on previous studies, capability of BCB+oocytes to develop to the blastocyst stage is higher than BCB-oocytes (Alm *et al*., 2005; Manjunatha *et al*., 2007; Opiela *et al*.,

Corresponding Author: Abd Wahid Haron, Department of Clinical Sciences, Faculty of Veterinary Medicine, University Putra Malaysia, 43400 Serdang, Selangor, Malaysia

2010) even after somatic cell nuclear transfer (Bhojwani *et al*., 2007). This competency of BCB+oocytes has been demonstrated on molecular and subcellular level as well (Torner *et al*., 2008). In addition, the BCB test selected a significantly higher number of competent oocytes in heifers for *in vitro* embryo production (Pujol *et al*., 2004). Majority of the genes upregulated in BCB+oocytes are related to cell cycle, transcription and protein biosynthesis regulation (Torner *et al*., 2008). On top of that, BCB- oocytes had lower transcript level of genes involved in mitochondrial biosynthesis (Opiela *et al*., 2010). So far however, no research has been found that studied the consequence of selection of developmentally competent oocytes on cryopreservation results. The objective of the present study was to evaluate the BCB test before vitrification and therefore enhance the maturity rate after vitrification of immature bovine oocyte.

MATERIALS AND METHODS

Reagents: Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Oocyte collection: Ovaries were collected from local abattoirs and transported to the laboratory within 3 h at 34-36°C in Phosphate-Buffer Saline (PBS; P-4417) containing penicillin-streptomycin (100,000 IU of penicillin and 100 mg of streptomycin per liter). Cumulus-Oocyte Complexes (COCs) were obtained by slicing method in tissue culture medium 199 (Medium199, 12340; Gibco) containing 25 mM HEPES, Earl's salts, L-glutamine and 2 mg mL^{-1} sodium bicarbonate modified by the addition of 4 mg mL^{-1} bovine serum albumin (BSA, fraction V, A-3311) and gentamycin 50 μg mL^{-1} (G-1264).

Brilliant Cresyl Blue test (BCB): Immediately after oocyte collection only oocytes with 4-5 layers of cumulus cells and homogenous cytoplasm were exposed to BCB (B-2002) diluted in mDPBS at a concentration of 26 μM for 90 min at 38.5°C in humidified air (Pujol *et al*., 2004; Alm *et al*., 2005).

After exposure to BCB, oocytes were washed two times in mDPBS and classified into two groups, depending on color of cytoplasm (Pujol *et al*., 2004; Alm *et al*., 2005): Blue Cytoplasm COCs (BCB+) and oocytes without Blue Cytoplasm coloration (BCB-). Control oocytes were not exposed to the stain and directly put into maturation solution.

Vitrification and warming: The vitrification protocol was adapted from Kuwayama *et al*. (2005). After separation of oocytes by BCB test, oocytes were washed two times in holding solution (HS, Hepes-buffered TCM medium supplemented with 20% Fetal Calf Serum, FCS) for about 15 min. Groups of four COCs were incubated in the first vitrification solution (VS1; 7.5% (v/v) DMSO and 7.5% (v/v) EG in HS) for 10-12 min and then transferred to the second vitrification solution (VS2; 15% (v/v) DMSO, 15% (v/v) EG and 0.5 mol L^{-1} (w/v) sucrose in HS) for a further 60 sec. Oocytes were immediatley loaded on Cryotop (Kitazato Supply Co, Fujinomiya, Japan) and submerged into Liquid Nitrogen (LN_2) for storage. The time of exposure from VS_2-LN_2 was <90 sec. All vitrified oocytes were maintained in LN_2 at least for 10 days. Immediately after removing the Cryotop storage device from LN_2, thin strip of Cryotop was submerged in 3 mL pre-warmed (39°C) HS plus 1 M sucrose (T1) and oocytes were detached smoothly from the Cryotop device. Immature oocytes were left in T1 for 1 min and then transferred to HS plus 0.5 and 0.15 M sucrose solution for 3 and 5 min, respectively. Finally, the immature oocytes were washed twice in HM for 5 min each and processed for *in vitro* maturation.

***In vitro* Maturation (IVM):** Immature oocytes were washed 2 times in medium 199 supplemented with 4 mg mL^{-1} BSA and washed again in maturation solution containing hepes-buffered medium 199 supplemented with 10% fetal calf serum, 0.2 mM sodium pyruvate (P-5280), 50 μL mL^{-1} Gentamycin (G-1264) and 1 μg mL^{-1} oestradiol-17β (E-8875). Approximately 20-30 oocytes were incubated in 400 μL of maturation solution under mineral oil in 4 well plates for 22-24 h at 38.5°C under 5% CO_2 atmosphere with high humidity. The vitrified BCB-test separated oocytes also underwent the same maturation process with control group after warming.

Nuclear maturation stage: For the purpose of maturation determination, 24 h after maturation, oocytes were denuded using 0.1% (w/v) hyaluronidase (Type 1-S) in Hepes-buffered medium 199 by vortexing. The cumulus-free oocytes were fixed in aceto-ethanol mixture (1:3, v/v) at 5°C for 24 h. Once fixed, oocytes in groups of 5-10 were mounted on slides. Cover-slip with 4 spots of silicon glued at the corners was gently pressed down until it touched and secured the oocytes. Afterward, aceto-lacmoid stain was passed under the cover-slip and remained there for 5 min followed by staining, a decolorizing solution (aceto-glycerol) was passed through to remove the stain residuals. Stained oocytes were examined under light microscope (400x) for nuclear stages determination.

Statistical analysis: All experiments were repeated 6 times. Significant differences among treatments were revealed by one-way analysis of variance followed by Duncan's multiple range test for mean comparisons ($p<0.05$) using SAS software ver. 9.1 (SAS Inst., Cary, NC).

RESULTS AND DISCUSSION

A representative number of immature oocytes (286 oocytes) after vitrification, warmed and cultured for IVM were assigned for determination of polar body rate and nuclear maturity. Higher number of oocytes in BCB+group had extruded first polar body compared with other vitrified groups but not significantly. This polar body rate was nevertheless, significantly lower than percentage recorded for control group Table 1 ($p<0.05$). Compared to the control group without any degenerated oocytes, the treatment groups significantly undergone degeneration ($p<0.05$) Table 1.

In BCB- oocytes, the rate of blocked oocytes at the GV stage was significantly higher than other treated oocytes. Only 1 out of 72 oocytes in control group were in GV stage and the rest progressing toward nuclear maturation. Vitrification of immature oocytes significantly reduced attaining nuclear maturity compared to control group 87 and 51%, respectively (Table 2). Within vitrified groups, oocytes reaching to the MII stage was significantly higher in BCB+group (51.5%) compared with BCB (27.9%) and vitrified-control (40.3%) groups.

The present study showed that staining of bovine immature oocytes with BCB stain recognized developmentally competent oocytes for vitrification of oocytes. Compare to the routine selection method based only on morphology (vitrified-control group), polar body and nuclear maturity was significantly enhanced by selection of BCB+oocytes.

In the present study, we have found that the percentage of oocytes that showed polar body and reached MII stage was significantly higher in BCB+oocytes compared to BCB-oocytes but significantly lower than control group (Table 1). This higher nuclear maturation rate is not surprising because BCB+ are developed and BCB-oocytes are developing or need some more time to complete their cytoplasmic needs for continuing development. In fact, Majority of the genes upregulated in BCB+oocytes are related to cell cycle, transcription and protein biosynthesis regulation (Torner *et al*., 2008) while BCB-oocytes had lower transcript level of genes involved in mitochondrial biosynthesis (Opiela *et al*., 2010).

The results are in agreement with earlier reports which have shown that the proportion of oocytes developed to MII stage in BCB + group was significantly higher than in BCB-group in bovine (Alm *et al*., 2005), buffalo (Manjunatha *et al*., 2007), porcine (Wongsrikeao *et al*., 2006), mice (Wu *et al*., 2007) and goats (Rodriguez-Gonzalez *et al*., 2002; Urdaneta *et al*., 2003).

The harmful cryopreservation procedure resulted in low nuclear maturity of treatment groups compared with control group. The hazardous parts of cryopreservation process which ultimately can be the cause of cell death is the risk of ice crystal formation in cytoplasm (Liebermann *et al*., 2002) as well as chilling injury (Vajta and Kuwayama, 2006).

Therefore, by employing vitrification method we have tried to minimize these risks. On the other hand, a designed solution that allows vitrification is highly concentrated and toxic for the oocytes. Therefore, to lessen this toxicity, the Cryotop method is used (Kuwayama *et al*., 2005). In this method, achieving extra-rapid cooling-warming rates bring this possibility to lower the concentration of vitrification solution. We have earlierly tested the toxicity of two vitrification solutions used for Cryotop and OPS methods and established that Cryotop solution is less toxic to immatured bovine oocytes (unpublished data).

The maturation rate for vitrified BCB+ and vitrified-control groups are higher than previously reported by Matsumoto *et al*. (2001), Cetin and Bastan (2006) and Yamada *et al*. (2007).

Table 1: Polar body rate of treatment groups after vitrification

Groups	N	PB+	PB-	Degenerated
Control	72	49.0 (36/72)^{a}	51.0 (36/72)^{c}	0^{c}
BCB+	71	23.9 (17/71)^{b}	68.67 (49/71)^{ab}	7.4 (5/71)^{b}
BCB-	68	17.5 (12/68)^{b}	60.5 (41/68)^{bc}	22.0 (15/68)^{a}
Vitrified-control	75	18.6 (14/75)^{b}	75.1 (56/75)^{a}	6.2 (5/75)^{bc}

Data were pooled from 6 replicates, $^{a-d}$ Values with different superscripts in the same column are significantly different ($p<0.05$)

Table 2: Nuclear maturity of treatment groups after vitrification

Groups	N	Unclassified	GV	GVBD-MI	MII
Control	72	6.2 (4/72)	1.1 (1/72)^{c}	5.7 (4/72)^{c}	87.0 (63/72)^{a}
BCB+	66	7.6 (5/66)	19.8 (13/66)^{b}	21.1 (14/66)^{ab}	51.58(34/66)^{b}
BCB-	53	8.6 (5/53)	46.6 (24/53)^{a}	17.0 (9/53)^{b}	27.9 (15/53)^{d}
Vitrified-control	70	7.1 (5/70)	28.4 (20/70)^{b}	21.4 (17/70)^{a}	40.3 (28/70)^{c}

Data were pooled from 6 replicates, $^{a-d}$Values with different superscripts in the same column are significantly different ($p<0.05$)

CONCLUSION

In conclusion, based on the current study, selection of immature oocytes using BCB stain may be effectively used before cryopreservation of immature oocytes to improve the results after fertilization.

ACKNOWLEDGEMENT

The researchers thank Mr. Pedram Kashiani for assisting in data analysis. This research was supported by a grant from the Agro-Biotechnology Institute, Ministry of Science, Technology and Innovation Malaysia (06-05 ABI AB003).

REFERENCES

Alm, H., H. Torner, B. Lhrke, T. Viergutz and I.M. Ghoneim *et al*., 2005. Bovine blast cyst development rate *in vitro* is influenced by selection of oocytes by brillant cresyl blue staining before IVM as indicator for glucose-6-phosphate dehydrogenase activity. Theriogenology, 63: 2194-2205.

Bhojwani, S., H. Alm, H. Torner, W. Kanitz and R. Poehland, 2007. Selection of developmentally competent oocytes through brilliant cresyl blue stain enhances blastocyst development rate after bovine nuclear transfer. Theriogenology, 67: 341-345.

Cetin, Y. and A. Bastan, 2006. Cryopreservation of immature bovine oocytes by vitrification in straws. Anim. Reprod. Sci., 92: 29-36.

Hadi, H., H. Wahid, O.A. Mazni, Y. Rosnina and M. Daliri *et al*., 2010. Effect of equilibration temperature on *in vitro* viability and subsequent embryo development of vitrified-warmed immature bovine oocytes. Am. J. Anim. Vet. Sci., 5: 71-75.

Hawk, H.W. and R.J. Wall, 1994. Improved yields of bovine blastocysts from *in vitro*-produced oocytes. I. Selection of oocytes and zygotes. Theriogenology, 41: 1571-1583.

Hazeleger, N.L., D.J. Hill, R.B. Stubbings and J.S. Walton, 1995. Relationship of morphology and follicular fluid environment of bovine oocytes to their developmental potential *in vitro*. Theriogenology, 43: 509-522.

Ishizaki, C., H. Watanabe, M.M.U. Bhuiyan and Y. Fukui, 2009. Developmental competence of porcine oocytes selected by brilliant cresyl blue and matured individually in a chemically defined culture medium. Theriogenology, 72: 72-80.

Kuwayama, M., G.B. Vajta, S. Ieda and O. Kato, 2005. Comparison of open and closed methods for vitrification of human embryos and the elimination of potential contamination. Reprod. Biomed. Online, 11: 608-614.

Liebermann, J., F. Nawroth, V. Isachenko, E. Isachenko, G. Rahimi and M.J. Tucker, 2002. Potential importance of vitrification in reproductive medicine. Biol. Reprod., 67: 1671-1680.

Manjunatha, B.M., P.S.P. Gupta, M. Devaraj, J.P. Ravindra and S. Nandi, 2007. Selection of developmentally competent buffalo oocytes by brilliant cresyl blue staining before IVM. Theriogenology, 68: 1299-1304.

Matsumoto, H., J.Y. Jiang, T. Tanaka, H. Sasada and E. Sato, 2001. Vitrification of large quantities of immature bovine oocytes using nylon mesh. Cryobiology, 42: 139-144.

Morato, R., D. Izquierdo, M.T. Paramio and T. Mogas, 2008. Cryotops versus Open-Pulled Straws (OPS) as carriers for the cryopreservation of bovine oocytes: Effects on spindle and chromosome configuration and embryo development. Cryobiology, 57: 137-141.

Opiela, J., D. Lipinski, R. Slomski and L. Katska-Ksiazkiewicz, 2010. Transcript expression of mitochondria related genes is correlated with bovine oocyte selection by BCB test. Anim. Reprod. Sci., 118: 188-193.

Pujol, M., M. Lpez-Bejar and M.T. Paramio, 2004. Developmental competence of heifer oocytes selected using the Brilliant Cresyl Blue (BCB) test. Theriogenology, 61: 735-744.

Rodriguez-Gonzalez, E., M. Lopez-Bejar, E. Velilla and M.T. Paramio, 2002. Selection of prepubertal goat oocytes using the brilliant cresyl blue test. Theriogenology, 57: 1397-1409.

Torner, H., N. Ghanem, C. Ambros, M. Hlker and W. Tomek *et al*., 2008. Molecular and subcellular characterisation of oocytes screened for their developmental competence based on glucose-6-phosphate dehydrogenase activity. Reproduction, 135: 197-212.

Urdaneta, A., A.R. Jimenez, D. Izquierdo and M.T. Paramio, 2003. Effect of the addition of glutathione and glucose to the culture medium on embryo development of IVM-IVF prepubertal goat oocytes. Zygote, 11: 131-138.

Vajta, G. and M. Kuwayama, 2006. Improving cryopreservation systems. Theriogenology, 65: 236-244.

Wongsrikeao, P., T. Otoi, H. Yamasaki, B. Agung and M. Taniguchi *et al*., 2006. Effects of single and double exposure to brilliant cresyl blue on the selection of porcine oocytes for *in vitro* production of embryos. Theriogenology, 66: 366-372.

Wu, Y.G., Y. Liu, P. Zhou, G.C. Lan and D. Han *et al*., 2007. Selection of oocytes for *in vitro* maturation by brilliant cresyl blue staining: A study using the mouse model. Cell Res., 17: 722-731.

Yamada, C., H.V.A. Caetano, R. Simoes, A.C. Nicacio and W.B. Feitosa *et al*., 2007. Immature bovine oocyte cryopreservation: Comparison of different associations with ethylene glycol, glycerol and dimethylsulfoxide. Anim. Reprod. Sci., 99: 384-388.