Evaluation of sows oocytes viability through Trypan Blue staining after vitrification

Simona MARC, C. MIRCU, Nicoleta CREȚAN, G. OTAVĂ, Camelia TULCAN, I. HUȚU
University of Agricultural Sciences and Veterinary Medicine of Banat "The King of Michael I" of Romania, Faculty of Veterinary Medicine, Timisoara, Romania
E-mail address: calinmircu@usab-tm.ro

Abstract

Along sperm and embryo cryopreservation that are used routinely also in animal assisted reproduction, studying are done also on animal oocyte cryopreservation in order to find the best conditions to preserve their viability. Vitrification is one of the methods that can be used in order to preserve oocytes. The higher reactive oxygen species that are formed during in vitro conditions can influence the success of assisted reproduction technique. The aim of this study was to evaluate the antioxidant potential of ascorbic acid (0.5mM) and rosmarinic acid (105µM) added in media for in vitro maturation on swine oocyte subjected to vitrification. COC's viability after vitrification was evaluated by 0.02% Trypan Blue staining. Comparing experimental groups C (vitamin C) and AR (rosmarinic acid) with group M (control), relative to the number of vitrified oocytes, a slight increase in their viability is observed, with 16.67% (C, class I) and 33.33% (AR, class II), respectively. Regardless of the treatment applied, the oocyte class is associated with viability (p = 0.048). Due to low number of oocytes used in each group we can concluded that supplementation of oocyte maturation media before vitrification with rosmarinic acid and ascorbic acid could produce a slight increase in viability.

***Key words**: antioxidants, oocyte, vitrification*

Introduction

Vitrification of oocytes and embryos is a revolutionary cryopreservation technique used both in human (Konc et al. 2014) and in animals (Somfai et al. 2014; Spricigo et al., 2015; Yang et al. 2002; El-Sokary et al., 2013) assisted reproduction techniques (ART) performed in diferent conditions and with different results.

Vitrification is defined as the ultrarapid solidification of a solution by an extreme elevation in viscosity at low temperatures without ice crystal formation (Konc et al., 2014).

The main causes of cell death during cryopreservation is ice crystal formation and toxic concentrations of solutes. In order to reduce the negative effects of cryopreservation, cryoprotective additives (CPA) are used. They are: *intracellular/membrane-permeating* (propyleneglycol, dimethyl sulfoxide, glycerol, ethylene glycol) and *extracellular* (sucrose, trehalose, glucose, amid, ficoll, proteins and lipoproteins). The first ones displaces water via an osmotic gradient and partly occupies the place of the intracellular water, while the extracellular cryoprotective additives increases the extracellular osmolarity generating an osmotic gradient across the cell membrane supporting the dehydration of the cell before cryopreservation and also prevents the rapid entry of water into the cell after thawing. During vitrification cells are dehydrated before the ultrarapid cooling by high concentration of CPA (Konc et al., 2014; Yang et al., 2002).

Oocytes are very sensitive to cryoprotectants used during cryopreservation protocols. Although vitrification of matured porcine oocytes has high survival rates, obtaining embryos by IVF or ICSI from them is difficult (Somfai et al., 2014). Cooling/warming processes from vitrification technique of porcine oocytes at MII stage determined accumulation of reactive oxygen species (ROS), parthenogenetic activation and spindle abnormalities (Somfai et al., 2014).

Sources of ROS during ART procedures could be either endogenously (immature spermatozoa, leukocytes, oocyte, cumulus mass cells, follicular fluid, embryos) or exogenous

environmental factors (visible light, culture media, pH, temperature, oxygen concentration, centrifugation, cryopreservation)(Mbemya et al., 2017; Agarwal et al., 2014). When is an imbalance between reactive oxygen species (ROS) and a biological system's ability to readly detoxify the reactive intermediates or repair the resulting damage, oxidative stress apears (Roychoudhury et al., 2017).

Because during *in vitro* conditions oocytes are separated from the body and do not benefit from maternal antioxidant protection, supplemented antioxidants are needed. Studies indicates that supplementing maturation media with different antioxidants such as β-mercaptoethanol (Sadeesh et al., 2014), cysteine, cysteamine (Beheshti et al., 2011), palm pollen grain extract (Salek-Abdollahi et al., 2015), quercetin and taxifolin – exogenous flavonoids (Kang et al., 2016), *Gundelia Tourneforii* leaves hydro alcoholic extract (Abedi et al., 2014) can improve oocytes maturation based on nuclear morphological changes.

Regarding vitamin C (L-ascorbic acid), a water-soluble antioxidant there are few studies to investigate it's antioxidant effects during *in vitro* maturation of oocytes (Sovernigo et al., 2017; Comizzoli et al. 2003; Tatemoto et al., 2001), most of studies were performed to emphasize it's beneficial role on freezing spermatozoa (Varo et al., 2014; Fanaei et al., 2014) or on improved motility and reduced DNA damage in post-thaw spermatozoa.

Another antioxidant used especially in freezing extenders which improved sperm quality after cryopreservation was rosmarinic acid (Luno et al., 2014; Luno et al., 2015; Olaciregui et al., 2017). Rosmarinic acid is one of the first secondary metabolites produced in plant cell cultures in extremely high yields, up to 19% of the cell dry weight. Other promising biological activities of rosmarinic acid and its derivatives (rabdosiin and lithospermic acid B) are: improvement of cognitive performance, prevention of the development of Alzheimer's disease, cardioprotective effects, reduction of the severity of kidney diseases and cancer chemoprevention. (Bulgakov et al.,2012).

The purpose of this present research was to evaluate the viability of swine oocyte after *in vitro* maturated in media supplemented with ascorbic acid and rosmarinic acid and cryopreserved through vitrification.

Materials and methods

Swine ovaries (n=10) were collected from slaughterhouse and transported to the laboratory in containers containing 0.9% NaCl solution supplemented with antibiotics (Pen/Strep), at 33-35^{0}C within two hours. Handling medium for COC (cumulus -oocytes- complexes) was Dulbecco-PBS (D-8662) supplemented with 100 µl Pen/Strep (17-602F, Lonza); 3.6 mg sodium piruvate, 30 mg BSA (A9647, Sigma-Aldrich), 100 mg glucose (G7021, Sigma-Aldrich). COCs were aspirated by puncture procedure from medium to large follicles with 18G needle attached to a 5 ml syringe.

Classification of COCs based on morphological aspects was done under stereomicroscope (Stemi 2000-C, ZEISS) with hot plate (33.4^{0}C) after criteria of Antosik et al. (2010) with minor modification: *Ist class* - CI (COCs with cumulus compact and unexpanded, with full or at least 5 layers of cumulus cells, cytoplasm clearly seen, dense and homogenous, *II^{md} class* – CII (COCs with cumulus compact, thick, 2-4 layers of cumulus cells, covering all of zona pellucida, cytoplasm dense, with uniform granulation) and *III^{d} class* - CIII (oocytes partially denuded of cumulus cells, or with 1-2 complete layers of cumulus cells and/or with irregular shrunken cytoplasm).

The maturation culture medium was prepared in our laboratory after Parrish et al. (1986) protocol with slight modifications: TCM 199 HEPES modification media, (M2520) with 10% ECS and 15 µl FSH (F8174, Sigma-Aldrich) - *group M* (control), in experimental groups we added ascorbic acid (0.5 mM) – *group C* and rosmarinic acid (105 µM) - *group AR*. Pools of 8-10 COCs

were maturated in 400µl media in 4 well dishes (Nunc, Germany) covered with mineral oil at 38.5^0C in 5% CO_2 humidified air atmosphere for 44h. After 44h of culture, all COC were examined for maturation, signs as expansion and mucification of cumulus cells were observed and were cryopreserved by vitrification according to there experimental group (M, C, AR group) and their morphological class (M1, M2, M3, C1, C2, C3, AR1, AR2, AR3).

Vitrification steps were: 15 min in Freezing 1 media (750 µl DMSO, 750 µl EG, 850 µl ECS and 7.65 ml TCM99), 1 min in Freezing 2 media (1500 µl DMSO, 1500 µl EG, 1.71g sucrose, 520 µl ECS, 5.23 ml TCM199), aspiration in 0.5 ml straws, sealed with MRSIDUAL V3 device (IMV) and imersed into liquid N_2 container, where they staid for 6 days.

Thawing steps were: 1 min, at 37^0C in a water bath, 1 min into Thawing 1 media (3.42g sucrose, 5.28 ml TCM199 and 1.30 ml FCS), 3 min into Thawing 2 media (1.9g sucrose, 6.64ml TCM199 and 1.65 ml FCS).

COC's viability after vitrification was evaluated by 0.02% Trypan Blue (T646, Sigma) staining, for 2 minutes.

Results and discussions

The results of viability evaluation of 57 swine *in vitro* maturated COCs and after vitrification, done by Trypan blue staining methods are presented in figure 1 and 2.

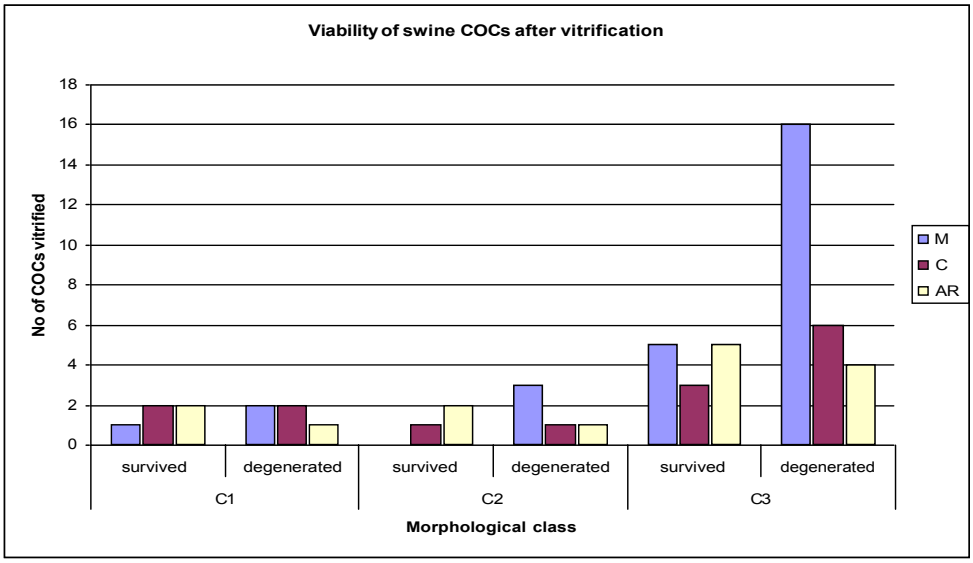

Figure 1. Classification of swine COC's after vitrification based on there viability

After thawing sow oocytes from the control group, 33.33% of the class I (M1) oocytes were viable and 66.66% non-viable, of the second class (M2) none was viable and in the third class oocytes (M3), 23.08% were viable and 71.19% were not viable.

Supplementation of the maturation media with vitamin C did not lead to an increase in the oocytes viability, regardless the morphological classification, thus class I (C1) and II (C2) 50% were viable and in the third class 33.33% (C3).

Choosing the vitamin C supplement as antioxidant for *in vitro* maturation media of swine COCs was based on other researcher's results. Comizzoli et al. (2003) observed that the compromised cat oocyte function during non-breeding season can be overridden by including supplemental FSH and antioxidants (0.5 mM vitamin C or cysteine) in maturation media. Kere et

al. (2013) testing various concentrations of vitamin C supplemented in IVM and IVC media of porcine oocytes and parthenotes and handmade cloned embryos observed that although nuclear maturation of oocytes was not affected by the addition of vitamin C, the intracellular glutathione levels were significantly increased and ROS reduced at 50 µg/ml vitamin C; added in IVC media, vitamin C improved blastocyst rates and total cell numbers and reduces apoptotic indices. In another study done on bovine oocytes, Sovernigo et al. (2017) suggest that antioxidants (vitamin C 50 µg/ml, quercetin 2µM, cyteamine 100µM, carnitine 0.5mg/ml or resveratrol 2µM) used during IVM may reduce oxidative stress which improved blastocyst development.

In COC's groups were we used rosmarinic acid as antioxidant we observed 66.66% of class I (AR1) and II (AR2) and 55.55% of COCs class III (AR3) survived after vitrification. Rosmarinic acid is used as an antioxidant in cryopreservation of semen. Olaciregui et al. (2017) proved for the first time that ovine spermatozoa freeze-drying in medium supplemented with 105 µM rosmarinic acid can be lyophilized effectively, stored at room temperature for long term, and even to starts embryo development after ICSI. Acid rosmarinic (105 µM) has beneficial effects also on boar semen cryopreservation were improves sperm parameters (total and progressive motility, viability, acrosome integrity) and sperm DNA integrity by reducing DNA oxidation rate (Luno et al., 2014; Luno et al., 2015). In swine reproduction, Somfai et al. (2014) reported the first successul piglet production from cryopreserved oocytes by vitrification.

Comparing groups C and AR with group M, relative to the number of vitrified oocytes, a slight increase in their viability is observed, with 16.67% (C1) and 33.33% (AR1), respectively.

The results obtained for the experimental groups (M, C and AR) require acceptance of the null hypothesis (Ho); the results does not support the hypothesis that treatment with antioxidants supports viability (Krusckal-Willis test, $p = 0.605$) or, on the contrary, causes mortality - unviability (Krusckal-Willis test, $p = 0.429$) of oocytes.

Regardless of the treatment applied, the oocyte class is associated with viability ($p = 0.048$) at statistically accepted thresholds. The classification of non-viable oocytes is close to the significance threshold (Krusckal-Willis test, $p = 0.059$).

Vitrification of oocytes can be done before or after *in vitro* maturation. Comparing these moments, Milovanov et al. (2016) concluded that cultivation of oocytes before vitrification brings more advantages for the meiotic resumption.

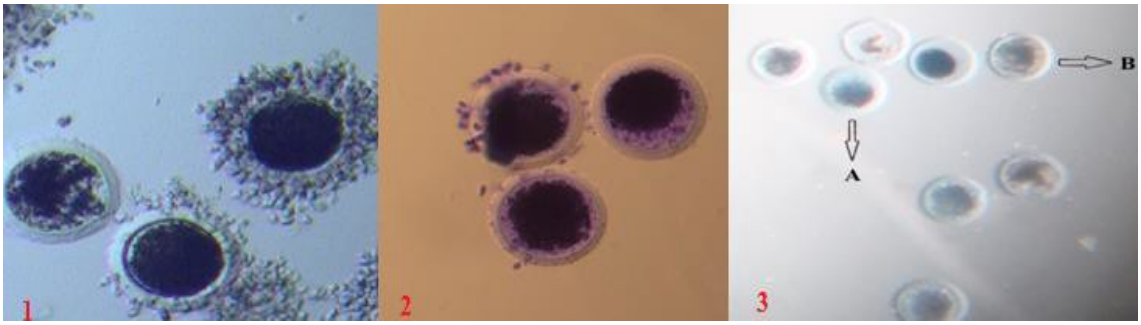

Figure.2 Live oocyte from group AR1 (1), dead oocytes from group M3 (2) and oocytes from group C3, A-dead oocyte, B-live oocyte (3)

Staining methods used in ART are very useful in order to see the quality of gametes and of embryos obtained. Some of them do not affect the viability of the cell, if are used correctly and for a short time (Brilliant Cresyl Blue, Hoechst 33342). Tripan blue (adiazo dye) is a supravital stain and an inexpensive marker that is used for studying cellular viability (membrane of live cell

is able to exclude the dye, whereas a nonviable cell will have a blue cytoplasm) (Melzer et al., 2016).

Testing the viability of the oocytes after cryopreservation is a mandatory step in order to see if the technique used had good results due to numerous factors implicated.

Further experiments are needed to clarify the effects of antioxidants on viability of the oocyte during vitrification and their capacity to sustain fertilization and developmental competence after thawing.

Conclusions

- Viability of oocytes depends on their class, while lack of viability is not necessarily associated with quality classes, so oocytes in C1 group had a higher viability of 16.67% and those in group AR by 33, 33% of the control group (M1).
- Supplementation of oocyte maturation media before vitrification with rosmarinic acid and ascorbic acid could determine a slight increase in viability, but this can not be supported statistically by our study.
- The use of Trypan blue to study oocytes viability is a quick, easy and efficient method

Aknowledgments

The research was carried in the IVF (In Vitro Fertilization) laboratory from the Horia Cernescu Research Unit equipped through POSCCE 2669 program

References

21. ABEDI, A.; ROUHI, L., PIRBALOUTI, A.G. (2014) - *Effect of Gundelia Tournefortii leaves extract on in immature mouse oocytes,* Journal of Herbal Drug, 5, 2, 84-89.
22. AGARWAL, A., DURAIRAJANAYAGAM, D., PLESSIS, S. (2014) - *Utility of antioxidants during assisted reproductive techniques: an evidence based review*, Reproductive Biology and Endocrinology, 12, 112, https://doi.org/10.1186/1477-7827-12-112.
23. ANTOSIK P., KEMPISTY B., JACKOWSKA M., BUKOWSKA D., LIANERI M., BRUSSOW K.P., WOZNA P., JASKOWSKI J.M. (2010) - *The morphology of porcine oocytes is associated with zona pellucida glycoprotein 3 and integrin beta 2 protein levels*. Veterinarni Medicina, 55, (4): 154–162.
24. BEHESHTI, R., MOHAMMADI-ROSHANDEH,A., GHALEHKANDI GIASI, J., GHAEMMAGHAMI, S., HOUSHANGI, A.F. (2011) - *Effect of antioxidant supplements on in vitro maturation of bovine oocyte,* Advances in Environmental Biology, 5, 7, 473-1475.
25. BULGAKOV, V.P., INYUSHKINA, Y.V., FEDOREYEV, S.A. (2012) – *Rosmarinic acid and its derivates: biotechnology and applications*, Crit Rev Biotechnol, 32, 3, 203-217.
26. COMIZZOLI,P., WILDT,D.E. PUKAZHENTHI, B.S. (2003) - *Overcoming poor in vitro nuclear maturation and developmental competence of domestic cat oocytes during the non-breeding season*, Reproduction, 126, 809-816.
27. EL-SOKARY, M., KANDIEL, M.M.M., MAHMOUD, K.Gh.M., ABOUEL-ROOS, M.E.A., SOSA, G.A.M. (2013) – *Evaluation of viability and nuclear status in vitrified mature buffalo oocytes*, Global Veterinaria, 10, 3, 297-302.
28. FANAEI H, KHAYAT S, HALVAEI I, RAMEZANI V, AZIZI Y, KASAEIAN A, MARDANEH J, PARVIZI MR, AKRAMI M. (2014) - *Effects of ascorbic acid on sperm motility, viability, acrosome reaction and DNA integrity in teratozoospermic samples*, Iranian J Reprod Med., 12: 103-110.
29. KANG, J.T., MOON, J.H., CHOI, J.I., PARK, S.J., KIM, S.J., SAADELDIN, I.M., LEE, B.C. (2016) - *Effect of Antioxidant Flavonoids (Quercetin and Taxifolin) on In vitro Maturation of Porcine Oocytes,* Asian Australas. J.Anim.Sci., 29, 3, 352-358.
30. KERE, M., SIRIBOON, C., LO, N., NGUYEN, N., JU, J. (2013) – *Ascorbic* Acid *Improves the Developmental Competence of Porcine Oocytes After Parthenogenetic Activation and Somatic Cell Nuclear Transplantation,* Journal of Reproduction and Development, 59:78-84
31. KONC, J., KANYO, KATALIN, KRISTON, RITA, SOMOSKOI, B., CSEH, S. (2014) – *Cryopreservation of embryos and oocytes in human assisted reproduction*, BioMed Research International, article ID 307268, http://dx.doi.org/10.1155/2014/307268

32. LUÑO V, GIL L, OLACIREGUI M, GRANDIA, j., ANSO, T., DeBLAS, I (2015) - *Fertilisation rate obtained with frozen-thawed boar semen supplemented with rosmarinic acid using a single insemination timed according to vulvar skin temperature changes*, Acta Veterinaria Hungarica, 63, 1, 100-109.
33. LUÑO V, GIL L, OLACIREGUI M, GONZÁLEZ N, JEREZ RA, DE BLAS I. (2014) - *Rosmarinic acid improves function and in vitro fertilising ability of boar sperm after cryopreservation*, Cryobiology, 69, 1, 157-62.
34. MBEMYA, G.T., VIEIRA, L.A., CANAFISTULA, F.G., LOIOLA PESSOA, O.D., RIBEIRO RODRIGUES,A.P. (2017) – *Reports on in vivo and in vitro contribution of medical plants to improve the female reproductive function,* Reprod Clim, (http://dx.doi.org/10.1016/j.recli.2016.11.002)
35. MELZER, S., NUNES, C.S.M., ENDRINGER, D.C., ANDRADE, T.U., TARNOK, A., LENZ, D. (2016) – *Trypan blue as an affordable marker for automated live-dead cell analysis in image cytometry*, Scanning, 9999, 1-7.
36. MILOVANOV, CORNELIA, BERA-LUTESCU, EMANUELA, ZARCULA SIMONA, KELLER, T., OTAVA, G., TULCAN, CAMELIA, HUTU, I., BONCA, GH., AHMADI, M., MIRCU, C. (2016) – *Vitrification of germinal vesicle and metaphase II swine oocytes*, Lucr Stiin Med Vet USAMV Iasi, Ed."Ion Ionescu de la Brad", 59, 174-178.
37. OLACIREGUI,M., LUNO, VICTORIA, DOMINGO, PAULA., GONZALEZ, NOELIA, GIL, LYDIA (2017) – *In vitro developmental ability of ovine oocytes following intracytoplasmatic injection with freeze-dried spermatozoa*, Scientific reports, 7, 1096, DOI:10.1038/s41598-017-00583-0, www.nature.com/scientificreports.
38. *PARRISH J.J., SUSKO-PARRISH J.L., LEIBFRIED-RUTLEDGE M.L., CRITSER E.S., EYESTONE W.H., FIRST N.L.* (1986) -Bovine in vitro fertilization with frozen-thawed semen, Theriogenology, 25, 591–600.
39. ROYCHOUDHUR, S., AGARWAL, A., VIRK, G., CHO, C-L., (2017) - *Potential role of green tea catechins in the management of oxidative stress-associated infertility,* http://dx.doi.org/10.1016/j.rbmo.
40. SAADESH, E., SHAH, F., BALHARA, A.K., THIRUMARAN, S. M. K., YADAV, S., Yadav, P. S., (2014) **-** *Effect of growth factor and antioxidant on in vitro maturation of oocytes and cleavage rates of in vitro produced Indian buffalo (Bubalus bubalis) embryos*, Veterinarski Arhiv, 84 ,5, 459-474).
41. SALEK-ABDOLLAHIF, BAHARARA J, NEJADSHAHROKHABADI KH, NAMVAR F, AMINI E. (2015) - *Effect of Phoenix dactylifera pollen grain on maturation of preantral follicles in mice NMRI mice*. J HerbMed Pharmacol.,4, 3, 93-97.
42. SOMFAI, T., YOSHIOKA, K., TANIHARA, F., KANEKO, H., NOGUCHI, J., KASHIWAZAKI, N., NAGAI, T., KIKUCHI, K. (2014) – *Generation of live piglets from cryopreserved oocytes for the first time using a defined system for in vitro embryo production*, PLoS ONE 9(5): e97731. doi:10.1371/journal.pone.0097731.
43. SOVERNIGO,T.C., ADONA, P.R., MONZANI, P.S., GUEMRA, S., BARROS, F., LOPES, F.G., LEAL, C (2017) – *Effects of supplementation of medium with different antioxidants during in vitro maturation of bovine oocytes on subsequent embryo production*, Reproduction in Domestic Animals, 52, 4, 561-569.
44. SPRÍCIGO JFW, DIÓGENES MN, LEME LO, GUIMARÃES AL, MUTERLLE CV, SILVA BDM, ET AL. (2015) - *Effects of Different Maturation Systems on Bovine Oocyte Quality, Plasma Membrane Phospholipid Composition and Resistance to Vitrification and Warming*. PLOS ONE 10(6): e0130164. doi:10.1371/ journal.pone.0130164.
45. TATEMOTO, H., OOTAKI, K., SHIGETA, K., MUTO,N. (2001) – *Enhancement of developmental competence after in vitro fertilization of porcine oocytes by tratment with ascorbic acd 2-O-α-glucoside during in vitro maturation,* Biology of Reproduction, 65, 6, 1800-1806.
46. VARO-GHIURU,F., MICLEA ILEANA, HETTIG,A., LADOSI,I., MICLEA, V., EGERSZEGI,I., ZAHAN, M. (2015) - *Lutein, trolox, ascorbic acid and combination of trolox with ascorbic acid can improve boar semen quality during cryopreservation*, CryoLetters, 36, 1, 1-7.
47. YANG, B.C., IM, G.S., CHANG, W.K., LEE, Y.K., OH, S.J., JIN, D.I., IM,K.S., LEE, C.K. (2003) – *Survival and in vitro developemnt of immature bovine oocytes cryopreserved by vitrification*, Asian-Aust.J.Anim. Sci, 16, 1, 23-28.