EFFECT OF CRYOPRESERVATION ON ATP CONTENT OF OVULATED RABBIT OOCYTES

Salvetti P.[1,2]*, Guérin P.[2], Joly T.[2]

[1]USC INRA/INSERM PrimaStem, INSERM U846, 18 Avenue du Doyen Lépine, 69675 Bron Cedex, France
[2]Université de Lyon, ENVL/ISARA LYON, unité CRYOBIO, 23 rue Jean Baldassini, 69364 Lyon Cedex 07, France
*Corresponding author: psalvetti@isara.fr

ABSTRACT

Oocytes cryopreservation represents a very promising tool for medicine and preservation of animal genetic resources. Assessment of oocytes viability after cryopreservation is often based on structural and developmental criteria whereas metabolism of thawed oocytes is not frequently studied. The aim of this study was to evaluate the impact of slow freezing and vitrification methods on the ATP concentration in ovulated rabbit oocytes. ATP contents of oocytes were evaluated by measurement of light emission intensity during the ATP-dependent oxidation of luciferin by luciferase. ATP levels were compared between frozen and vitrified oocytes after 2 hours of *in vitro* culture and fresh oocytes as a control. Independently of the cryopreservation method used, ATP content per cell was significantly ($P<0.001$) lower for cryopreserved oocytes as compared to fresh ones (6.34±0.39 pmol of ATP per fresh oocyte). Frozen oocytes showed a significantly ($P<0.001$) lower ATP metabolism than the vitrified ones (2.93±0.23 and 4.88±0.21 pmol of ATP per frozen and vitrified oocyte respectively). In conclusion, this study shows that ATP assay is an attractive tool to evaluate the viability of cryopreserved oocytes. Moreover, according to these results, vitrification seems to be a more suitable method than slow freezing to cryopreserve ovulated rabbit oocytes.

Keywords*:* Rabbit, Oocytes, Cryopreservation, ATP, Bioluminescence.

INTRODUCTION

Oocytes cryopreservation represents one of the most exciting challenges for cryobiologists. It could be an effective tool for the preservation of women fertility thereby avoiding embryo manipulation and all legal and ethical problems involved. Moreover, oocytes cryopreservation would be a very interesting tool to preserve animal genetic biodiversity in addition to semen freezing, particularly for the preservation of rare and endangered species (Shaw *et al.*, 2000).

In rabbits, the conservation of genetic diversity is only permitted by the standard embryo freezing method at the compacted morulae stage (Joly *et al.*, 1998). Since the extension of the French national cryobanking programs to rabbit species in 2003, more than 17,000 embryos issued from about fifty different populations were stored in liquid nitrogen (LN_2). Currently, oocytes cryopreservation is not used for the conservation of rabbit populations because the fertility obtained after thawing is very poor whether using slow freezing (Al Hasani *et al.*, 1989; Siebzehnruelb *et al.*, 1989; Vincent *et al.*, 1989) or vitrification (Cai *et al.*, 2005). These results can be explained by oocytes high sensitivity to cold temperatures, by oocytes morphological characteristics and by the lack of knowledge on oocytes maturation and fertilization (Fabbri *et al.*, 2000; Gosden, 2005; Gardner *et al.*, 2007; Shaw *et al.*, 2000). An additional problem in the study of oocytes cryopreservation lies in the evaluation of oocytes viability after thawing considering spindle and cytoskeleton damages (Vincent *et al.*, 1989; Cai *et al.*, 2005), *zona hardening* phenomenon (Fuku *et al.*, 1995; Larman *et al.*, 2006; Matson *et al.*, 1997) or fertilizing and developmental abilities *in vivo* or *in vitro*.

Currently, no study has evaluated the impact of cryopreservation procedures on the oocytes ATP metabolism. Several studies have shown that under conditions of mechanical and chemical stress, cells

release ATP more intensively in the extracellular environment by a mechanism remaining unknown (Joseph *et al.*, 2003; Lazarowski *et al.*, 2003). Once ATP leaves the oocyte, it is rapidly degraded by ecto-ATPases on the cell surface (Schwiebert and Zsembery, 2003). Assessment of cellular ATP contents may thus provide useful information regarding the metabolic state and the integrity of the cell. The intensity of luminescence is measured by a luminometer or scintillation counter and is closely correlated to the ATP content of the cells. Evaluation of ATP content was used in a variety of experiments on human (Slotte *et al.*, 1990), porcine (Herrick *et al.*, 2003) and bovine (Rieger, 1997; Stojkovic *et al.*, 2001; Tamassia *et al.*, 2004) oocytes. Theses studies have underlined that, independently of the considered species, ATP content in oocytes vary from 0.85 to 2,8 pmol depending on experimental conditions, stage of maturation, physiological status of the donor and method used.

The objective of our study was to evaluate the effect of slow freezing and vitrification on ATP contents of ovulated rabbit oocytes.

MATERIALS AND METHODS

Oocytes collection

This study was performed using fertile does from commercial genotype that we superovulated with a commercial gonadotrophin preparation (Stimufol®, Reprobiol, Belgium). Just before injecting, the hormones were dissolved in 56 ml of a physiological saline solution to a final concentration of 9 mg/ml of porcine Follicle Stimulating Hormone. Sixty hours before planned ovulation induction, the does were injected (s.c.) twice daily (12 h intervals) with different doses of the hormonal preparation during 2.5 days: twice 0.5 ml on day 1, twice 1 ml on day 2 and only one injection of 0.5 ml on morning of day 3.

Cumulus-oocyte complexes (COCs) were obtained after slaughtering of the donor does at 13 to 15 hours after ovulation induction by an intramuscular injection of 1.6 µg of buserelin acetate (Receptal®, Intervet, France). Genital tracts were immediately removed and COCs were recovered into a Petri dish by normograde perfusion of each oviduct with 20 mL of Euroflush® solution (IMV Technologies, France). COCs were counted and morphologically evaluated under stereoscopic microscope control (G x 10). Only COCs with homogeneous cytoplasm and compacted and multilayered cumulus were kept for the experiment. The cumulus cells were removed from the oocytes mechanically after exposure to 0.5% hyaluronidase (H4272, Sigma, France) in PBS. The corona radiata cells were not removed in order to limit excessive oocytes manipulation which can induce stress-induced ATP release by the cells. After washing 3 times in base medium constituted of Hepes-TCM199 (M7528, Sigma, France) + 20% Fetal Bovine Serum (F9665, Sigma, France), oocytes of each female were randomly assigned to three experimental groups: control (fresh oocytes), slow freezing (frozen oocytes), and vitrification group (vitrified oocytes).

Oocytes cryopreservation

Rabbit oocytes were incubated in medium on a warming plate at 37°C, protected from light until their cryopreservation according to two different protocols. All cryopreservation procedures including thawing step were performed at 37°C. For both cryopreservation methods, straws were stored in the LN_2 for at least 48 h.

"*Vitrification group*" This method was adapted from the report of Cai *et al.* (2005) with slight modifications. In a first step, oocytes were put in base medium supplemented with 10% (v/v) ethylene glycol ("EG"; E9129, Sigma, France) and 10% (v/v) Dimethyl Sulfoxide (Hybrimax® DMSO, Sigma, France) for 2 min before their transfer in the vitrification solution made of 20% EG, 20% DMSO, 0.65 mol/l Trehalose (T0167, Sigma, France) and 10 mg/ml Ficoll70 (F2878, Sigma, France) in base medium. Oocytes were quickly mounted in Open Pulled Straw (19050/0050, Minitübe, Germany) by

capillarity and plunged directly in LN_2 within 35 sec. For thawing, the OPS straws containing the oocytes were directly emptied into a bath containing 0.5 mol/l Trehalose in base medium during 5 min. The oocytes were incubated successively in three baths containing respectively 0.3, 0.1 and 0 mol/l Trehalose in base medium at 5 min intervals.

"*Slow freezing group*" This protocol was adapted from previous studies (Al Hasani *et al.*, 1989; Siebzehnruelb *et al.*, 1989; Vincent *et al.*, 1989) with some modification. Briefly, oocytes were incubated for 15 min into a solution containing 1.5 mol/l 1,2-Propanediol ("PROH"; 134368, Sigma, France) in base medium. Then, oocytes were placed in the freezing solution including 1.5 mol/l 1,2PROH and 0.2 mol/l Trehalose in base medium for 10 min and were mounted in 0.25 ml sterile French mini straw (ref n°006431, IMV, France) sealed by a sterile plug between two air bubbles. Then, the straws were placed into a programmable freezer (Cryocell 1200, IMV Technologies, France) equilibrated at -7°C. After a 5 min equilibration period, the seeding was performed by touching the straw near the sterile plug with a LN_2 pre-cooled forceps until the apparition of the first ice crystals. A second period of 10 min equilibration was used after the seeding. Then, the embryos were cooled down to -30°C at a freezing rate of 0.5°C/min before plunging the straws into LN_2. Straws were thawed in ambient air for approximately 10-15 sec before plunging into a water bath at 20°C until the disappearance of ice crystals (about 1 minute). After thawing, oocytes were washed in different baths during 5 min at room temperature in order to remove the cryoprotectant (1,2PROH) and rehydrate the cells. So, the embryos were placed successively in base medium containing 1 mol/l 1,2PROH + 0.2 mol/l Trehalose, 0.5 mol/l 1,2PROH + 0.2 mol/l Trehalose and 0.2 mol/l Trehalose.

ATP assays

Preliminary works have underlined that 2 hours of incubation at 38°C under 5% CO_2 in humidified atmosphere is the optimal time for ATP assessment in cryopreserved oocytes. ATP assessments were performed just after collection for fresh oocytes and after 2 hours of incubation for cryopreserved oocytes.

Concentrations of ATP were determined using the bioluminescent somatic cell assay kit FL-ASC (Sigma, France) as previously described by Stojkovic *et al.* (2001). Briefly, samples were rinsed three times in base medium before being transferred in PBS (P4417, Sigma, France) by groups of four to five oocytes. Then, 50 µl of PBS containing the oocytes were transferred into 5 ml plastic tubes equilibrated on ice (0°C) and 100 µl of ice-cold somatic cell reagent (FL-SAR) were added to all tubes and the mix was incubated for another 5 min on ice cold water. Subsequently, 100 µl of ice-cold assay mix (dilution 1:25 with ATP assay mix dilution buffer, FL-AAB) was added, and the tubes were kept for 5 min at room temperature in the darkness to avoid the flash reaction. Then, ATP content of the samples was measured using a luminometer (LB 9509 Junior; Berthold France SAS) with high sensitivity (0.01 pmol). In order to have simultaneous measurements of sample luminescence, tubes with oocytes were all measured once and then a second measure was performed in the reverse order. The mean of these two values expressed in Relative Light Unit was recorded to establish our final value. An eight-point standard curve (0-10 pmol/tube) was routinely included in each assay. The ATP content was determined by the formula derived from the linear regression of the standard curve and the luminescence values. For each modality, a minimum of four ATP assay repetitions were performed.

Statistical Analysis

The effect of cryopreservation treatment (3 levels) on ATP concentrations in fresh, frozen and vitrified oocyte was evaluated using analysis of variance (ANOVA) with one fixed effect (Statview®, SAS Institute Inc.). Means are presented ± standard error of the mean (S.E.M.) and are considered significantly different when p values are inferior to 0.05. Figure 1 shows the ATP levels for frozen and vitrified oocytes after 2 hours of culture and fresh oocytes. In comparison with other reports on ATP assays in fresh oocytes of different species, values obtained are higher (Herrick *et al.*, 2003; Rieger, 1997; Slotte *et al.*, 1990; Stojkovic *et al.*, 2001).

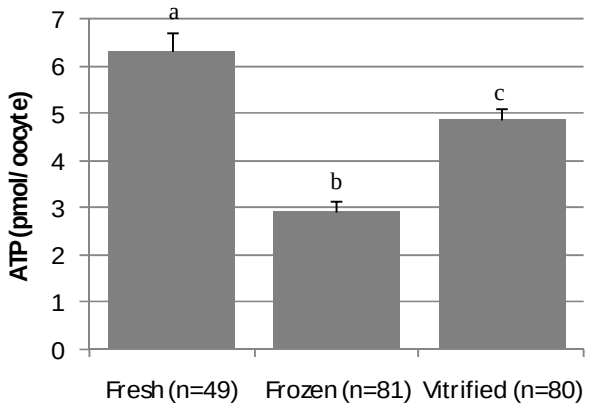

Figure 1: Comparison of ATP content between fresh, frozen and vitrified oocytes. Different letters (a, b, c) indicate significant differences (P<0.05)

RESULTS AND DISCUSSION

Unfortunately, no previous report has mentioned the ATP content of rabbit fresh oocytes, but the difference in ATP levels is probably due to the presence of *corona radiata* cells around the oocytes. So, either the cumulus cells have a non negligible ATP content or these oocytes have higher concentrations than the denuded ones. To remove the corona radiata cells, excessive manipulations are necessary which probably involve a lot of stress-released ATP rapidly degraded by the oocytes before assays (Joseph *et al.*, 2003; Lazarowski *et al.*, 2003). Additional experiments will be necessary to evaluate the mean ATP content of corona radiata cells and denuded oocytes in rabbit species.

Independently of the method used, the cryopreservation has a significant effect (P<0.001) on the ATP level of fresh *in vivo* matured rabbit oocytes (6.3±0.4 pmol of ATP per fresh oocyte). This observation underlines that the cryopreservation process affects the ATP metabolism of oocytes and suggest that mitochondria could be altered by exposition to cryoprotective agents or temperature variation including ice crystals formation. Moreover, a significant difference in ATP level was observed between frozen and vitrified oocytes after thawing and 2 hours of incubation (respectively 2.9±0.2 and 4.9±0.2 pmol of ATP per frozen and vitrified oocyte). So, we can assume that frozen oocytes have been altered during the interval between their recovery and their freezing, while waiting on the warming plate at 37°C (up to 2 hours), in opposition to vitrified oocytes which were cryopreserved just after their recovery. Additional measurements have been performed showing that the incubation time of fresh oocytes on a warming plate at 37°C have no significant effect on the ATP metabolism, hereby dismissing the hypothesis described above (data not shown).

According to this evaluation tool of oocytes viability, vitrification seems to be more adapted to cryopreservation of mature rabbit oocytes than slow freezing. In opposition to slow freezing, the absence of ice crystals formation during vitrification process decreases the risk of mechanical alterations of cellular structures. Further studies are required to evaluate the ultrastructural lesions in mitochondria of frozen and vitrified oocytes to establish a cause-effect relation between cellular damages and the ATP production in thawed oocytes.

CONCLUSIONS

This study is the first experiment performed with the objective to compare equilibrated freezing and vitrification for the cryopreservation of *in vivo* matured oocytes in rabbit species. According to other reports (Ding *et al.*, 2007; Gardner *et al.*, 2007; Kuwayama, 2007), vitrification seems to be the most promising method to preserve oocytes allowing the formation of a glassy state without ice crystals. Moreover, our results suggest that ATP assay using bioluminescence represents a useful tool to evaluate the viability of oocytes after thawing. Determination of ATP content can be used as a

metabolic indicator in addition to of morphological, structural and developmental criteria. This study is a preliminary work which should be completed by additional experiments investigating the spindle configuration, the *zona hardening* phenomenon or the *in vivo* developmental capacity after fertilization of cryopreserved oocytes at different incubation time after thawing.

ACKNOWLEDGEMENTS

This work was supported by the French National Institute of Agronomical Research – P.H.A.S.E. department. The authors thank gratefully Arne Lundin (Biothema AB, Sweden) and Manoel Tamassia (Univervisity of Illinois, United States) for their invaluable advices.

REFERENCES

Al-Hasani S., Kirsch J., Diedrich K., Blanke S., Van der Ven H., Krebs D. 1989. Successful embryo transfer of cryopreserved an in vitro fertilized rabbit oocytes. *Human Reprod., 4, 77-79.*

Cai X.Y., Chen G.A., Lian Y., Zheng X.Y., Peng H.M. 2005. Cryoloop vitrification of rabbit oocytes. *Human Reprod., 20, 1969-1974.*

Ding D.C., Ko C.S., Chu T.W., Chu Y.N., Chen I.C., Chen W.H., Wu G.T. 2007. Changes to the meiotic spindle and zona pellucida of mature mouse oocytes following different cryopreservation methods. *Anim. Reprod. Sci. (In press).*

Fabbri R., Porcu E., Marsella T., Primavera M.R., Rochetta G., Ciotti P.M., Magrini O., Seracchioli R., Venturoli S., Flamigni C. 2000. Technical aspects of oocyte cryopreservation. *Mol. Cell. Endocrinology, 169, 39-42.*

Fukku E., Xia L., Downey B.R. 1995. Utrastructural changes in bovine oocytes cryopreserved by vitrification. *Cryobiology, 32, 139-156.*

Gardner D.K., Sheehan C.B., Rienzi L., Katz-Jaffe M., Larman M.G. 2007. Analysis of oocyte physiology to improve cryopreservation procedures. *Theriogenology, 67, 64-72.*

Gosden R.G. 2005. Prospects for oocyte banking and in vitro maturation. *Journal of the National Cancer Institute Monographs, 34, 60-63.*

Herrick J.R., Brad A.M., Krisher R.L., Pope W.F. 2003. Intracellular adenosine triphosphate and glutathione concentrations in oocytes from first estrous, multi-estrous and testosterone treated gilts. *Anim. Reprod. Sci., 78, 123-131.*

Joly T., de Rochambeau H., Renard J.P. 1998. Etablissement d'une cryobanque d'embryons pour la conservation ex situ de la diversité génétique chez le lapin: aspects pratiques. *Genetique Selection Evolution, 30, 259–269.*

Kuwayama M. 2007. Highly efficient vitrification for cryopreservation of human oocytes and embryos: the cryotop method. *Theriogenology, 67, 73-80.*

Joseph S.M., Buchakjian M.R., Dubyak G.R. 2003. Colocalization of ATP release sites and ecto-ATPase activity at the extracellular surface of human astrocytes. *J. Biol. Chem., 278, 23331-23342.*

Larman M.G., Sheehan C.B., Gardner D.K. 2006. Calcium free vitrification reduces cryoprotectant-induced zona pellucida hardening and increases fertilization rates in mouse oocytes. *Reproduction, 131, 53-61.*

Lazarowski E.R., Boucher R.C., Harden T.K. 2003. Mechanisms of release of nucleotides and integration of their action as P2X- and P2Y-receptor activating molecules. *Mol. Pharmocol., 64, 785-795.*

Matson P.L., Graefling J., Junk S.M., Yovich J.L., Edirisinghe W.R. 1997. Cryopreservation of oocytes and embryos: use of a mouse model to investigate effects upon zona hardness and formulate treatment strategies in an *in-vitro* fertilization programme. *Human Reprod., 12, 1550-1553.*

Rieger D. 1997. Batch analysis of the ATP content of bovine sperm, oocytes, and early embryos using scintillation counter to measure the chemiluminescence produced by luciferin-luciferase reaction. *Analytical Biochemistry, 246, 67-70.*

Schwiebert E.M., Zsembery A. 2003. Extracellular ATP as a signaling molecule for epithelial cells. *Biochimica et Biophysica Acta, 1615, 7-32.*

Shaw J.M., Oranratnachai A., Trounson A.O. 2000. Fundamental cryobiology of mammalian oocytes and ovarian tissue. *Theriogenology, 53, 59-72.*

Siebzehnruelb E.R., Todorow S., Van Uem J., Koch R., Wildt L., Lang N. 1989. Cryopreservation of human and rabbit oocytes and one-cell embryos: a comparison of DMSO and propanediol. *Human Reproduction, 4, 312-317.*

Slotte H., Gustafson O., Nylund L., Pousette A. 1990. ATP and ADP in human pre-embryos. *Human Reprod., 5, 319-322.*

Stojkovic M., Machado S.A., Stojkovic P., Zakhartchenko V., Hutzler P., Gonçalves P.B., Wolf E. 2001. Mitochondrial distribution and adenosine triphosphate content of bovine oocytes before and after in vitro maturation; correlation with morphological criteria and developmental capacity after in vitro fertilization and culture. *Biol. Reprod., 64, 904-909.*

Tamassia M., Nuttinck F., May-Panloup P., Reynier P., Heyman Y., Charpigny G., Stojkovic M., Hiendleder S., Renard J.P., Chastant-Maillard S. 2004. In Vitro Embryo Production Efficiency in Cattle and Its Association with Oocyte Adenosine Triphosphate Content, Quantity of Mitochondrial DNA, and Mitochondrial DNA Haplogroup. *Biology of Reproduction, 71, 697-704.*

Vincent C., Garnier V., Heyman Y., Renard J.P. 1989. Solvent effects on cytoskeletal organization and in vivo survival after freezing of rabbit oocytes. *J. Reprod. Fertil., 87, 809-820.*