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The Effect of Vitrification and *in vitro* Culture on the Adenosine Triphosphate Content and Mitochondrial Distribution of Mouse Pre-Implantation Embryos

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ABSTRACT

Background: The mitochondria are an important source of adenosine triphosphate (ATP) production in pre-implantation embryo. Therefore, the objective of this study was to investigate the effect of vitrification and *in vitro* culture of mouse embryos on their mitochondrial distribution and ATP content. **Methods:** The embryos at 2-PN, 4-cell and blastocyst stages were collected from the oviduct of stimulated pregnant mice and uterine horns. Then, the embryos were vitrified with the cryotop method using ethylene glycol and dimethylsulphoxide. After evaluating the survival rates of vitrified embryos, their development to hatching stages were assessed. The ATP content of collected *in vivo* and *in vitro* embryos at different stages was measured by luciferin-luciferase bioluminescence assay. The distribution of mitochondria was studied using Mito-tracker green staining under a fluorescent microscope. **Results:** The survival rates of vitrified embryos at 2-PN, 4-cell and early blastocyst stages were 84.3, 87.87 and 89.89%, respectively. The hatching rates in previous developmental stages in vitrified group were 57.44, 66.73 and 70.89% and in non-vitrified group were 66.32, 73.25 and 75.89%, respectively (P>0.05). The ATP content of *in vivo* or *in vitro* collected embryos was not significantly different in both vitrified and non-vitrified groups (P>0.05). Mitochondrial distribution of vitrified and non-vitrified 2-PN embryos was similar, but some clampings or large aggregation of mitochondria within the vitrified 4-cell embryos was prominent. **Conclusions:** Vitrification method did not affect the mouse embryo ATP content. Also, the cellular stress was not induced by this procedure and the safety of vitrification was shown. *Iran. Biomed. J. 17 (3): 123-128, 2013*

Keywords: Mitochondria, Vitrification, Adenosine triphosphate (ATP)

INTRODUCTION

During the last decades, some improvements were done in vitrification techniques including increase in cooling rate and decrease in the volume of cryoprotectant by changing carrier system [1, 2]. Vitrification of pre-implantation embryos has been employed with high survival and developmental rates and it is a suitable alternative to slow-cooling technique [2-4].

Despite of the vast progression in vitrification techniques, some studies have reported that the vitrification procedures cause several structural and biochemical changes that may lead to loss of embryo viability or embryo quality [5-7]. It was also shown that the embryo vitrification at different developmental stages could affect their subsequent development [8, 9]. Special events during development of embryo, including pronucleus formation, syngamy, embryonic genome activation, successive cell cleavages, compaction, lineage differentiation and blastocoel formation are dependent adenosine triphosphate (ATP) [10]. Therefore, the mitochondria, as a source of ATP production, might be a critical factor in develop-mental competence of embryo and it is mainly localized in the region where needs more energy requirements [11].

Some investigations have shown that the embryonic cryopreservation cause some changes in mitochondrial distribution and ultrastructure and these alterations could decrease developmental competence of embryos [12- 14]. Moreover, *in vitro* culture condition could affect the metabolism and developmental capacity of mammalian embryos especially after vitrification and warming. It has been also shown that the mitochondria of embryo are sensitive to the culture condition [15-18]. During the embryo culture, the sufficient ATP

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content needs embryo development [19].

Therefore, cryopreservation procedure and culture condition may influence on the embryonic mitochondria and ATP content and to our best knowledge, poor information is available regarding to this subject. The aim of this study was to investigate the effect of vitrification and *in vitro* culture of mouse embryos at different developmental stages on the embryonic ATP content and mitochondrial distribution.

MATERIALS AND METHODS

Animals. Adult female (6-10 weeks old) and male (8-12 weeks old) NMRI mice were cared and used according to the guide for the care and use of laboratory animals of Tarbiat Modares University, Tehran, Iran. The mice were housed under a 12 h light:12 h dark regimen with a temperature of 23°C and relative humidity of 44%.

Experimental Design.

Experiment 1. To evaluate the effect of vitrification and warming procedure on the embryo development, ATP content and mitochondrial distribution were assessed. After collecting at 2-PN, 4-cell and early blastocyst stages, embryos were randomly divided into non-vitrified and vitrified groups and their development to hatching blastocysts was assessed. The ATP content of 2-PN, 4-cell and early blastocyst and mitochondrial distribution of 2-PN and 4-cell vitrified-warmed embryos were compared with non-vitrified control groups.

Experiment 2. To evaluate the effect of *in vitro* culture on the ATP of embryos, the ATP content of collected embryos at 4-cell and blastocyst stages in vitrified and non-vitrified embryos were analyzed.

Reagents and culture media. All reagents were purchased from Sigma Aldrich (Germany) except pregnant mare serum gonadotropin (Folligon; Intervent, Australia) and human chorionic gonadotropin hormone (Sereno, Switzerland). The medium used for isolation and culture of embryos was global medium total (Life Global, USA)

Embryo collection. The adult mice were superovulated using i.p. injection of 10 IU pregnant mare serum gonadotropin, which was followed 48 h later with another i.p. injection of 10 IU human chorionic gonadotropin hormone. After stimulating, the female mice were mated with fertile males. The presence of vaginal plug was checked on the next day and this day was designated as day 1 of gestation. The embryos at 2-PN, 4-cell and blastocyst stages were collected from oviduct and uterine horns of pregnant mice at 12-16, 38-40 and 84-86 h after human chorionic gonadotropin hormone injection, respectively.

Vitrification and warming. Embryos were vitrified by cryotop method described by Kuwayama [20]. Briefly, the embryos were transferred into equilibration solution, containing 7.5% DMSO, 7.5% ethylene glycol and 20% human serum albumin (HSA) in Ham's F10 at room temperature for 7 min. Then, the embryos were placed in vitrification solution, containing 15% DMSO, 15% ethylene glycol, 0.5 mol sucrose and 20% HSA in Ham's F10 for 1 min. The embryos were loaded to cryotop with a small volume of vitrification solution (<1.0 µL) and then plunged into liquid nitrogen. For warming, the cryotop was immersed into a solution of 1 mol sucrose containing 20% HSA in Ham's F10 at 37°C for 1 min. Afterward, the embryos were sequentially transferred into a solution of 0.5 mol sucrose for 3 min as well as a solution of 0.25 mol sucrose for 3 min and washed with a medium supplemented with 20% HSA. After 1 h of equilibration, the survival rate of embryos was assessed.

Assessment of in vitro embryo development. Vitrified and non-vitrified embryos at 2-PN (n = 192), 4-cell (n = 198) and blastocyst (n = 170) stages were cultured in 20-µL droplet of global media under mineral oil to hatching stage in 5% CO_2 in air at 37°C for 120, 72 and 24 h, respectively. The embryos were cultured in three groups and observed daily under an inverted microscope. Then, their developmental rates were recorded until hatching stage.

ATP assay. The ATP content of the embryos was measured using the method described previously [21]. Briefly, the non-vitrified and vitrified embryos at different developmental stages were individually frozen at -80°C in 20 µL of ultrapure water (n = 20 in each group of study). ATP levels were quantified by measuring the luminescence (Berthold LB 9501 luminometer, Germany) generated in an ATP-dependent luciferin-luciferase bioluminescence assay (Bioluminescence Somatic Cell Assay System; Sigma, USA). A standard curve with different ATP concentrations was generated for each series of analyses. The ATP content was determined from the formula for the standard curve (Lineweever-Burk Plot).

Table 1. The survival and developmental rates of vitrified and non-vitrified mouse embryo at different developmental stages

Stage of embryo	Group	No. of embryos	No. of survived (%)	No. of blastocyst (%)	No. of hatched (%)
2-PN	control	90	90 (100.00)	71 (79.73)	56 (66.32)
2-PN	vitrified	102	86 (84.30)	62 (74.50)	48 (57.44)
4-cell	control	99	99 (100.00)	87 (89.96)	72 (73.25)
4-cell	vitrified	99	87 (87.87)	68 (82.40)	55 (66.73)
blastocyst	control	71	71 (100.00)	-	54 (75.89)
blastocyst	vitrified	99	89 (89.89)	-	64 (70.89)

Visualization of the mitochondria using Mito-tracker green. The presence of viable mitochondria was identified by Mito-tracker green (Molecular Probes, Invitrogen, Eugene, Oregon, USA) staining described before by Van Blerkom *et al.* [22] with some modifications. Stock solution of Mito-tracker green at a concentration of 1 mmol was prepared in DMSO and stored at -20°C. The fresh or non-vitrified and vitrified-warmed embryos at 2-PN and 4-cell stages (n = 10 in each groups of the study) were stained in PBS with Mito-tracker Green (0.2 mmol) at 37°C for 10 min. After washing the samples in PBS, the embryos were visualized using a fluorescent microscope at 490 wavelengths.

Statistical Analysis. All experiments were repeated at least four times. The developmental rates of embryos were analyzed by one-way ANOVA and Tukey's HSD was used as post hoc tests. ATP levels were analyzed statistically by the *t*-test. Statistical significance was described as $P<0.05$.

RESULTS

The survival and developmental rates of embryos at different developmental stages have been shown in Table 1. The survival rates of vitrified embryos at 2-PN, 4-cell and early blastocyst stages were 84.3, 87.87 and 89.89%, respectively and there was no significant difference in survival rate at different developmental stages ($P>0.05$). The rate of blastocyst formation in 2-PN and 4-cell embryos in control group was 79.73 and 89.96% and in vitrified group, was 74.50 and 82.40%, respectively. The hatching rate of embryos at 2-PN, 4-cell and blastocyst stages in vitrified group were 57.44, 66.73 and 70.89% and in non-vitrified samples were 66.32, 73.25 and 75.89%, respectively. There were not statistically significant different in blastocyst and hatching rates between vitrified and non-vitrified groups ($P>0.05$).

The ATP content of the vitrified and non-vitrified mouse embryo. The ATP content of mouse embryos at different developmental stages were obtained from *in vivo* condition and compared in vitrified-warmed and non-vitrified samples (Fig. 1). The levels of embryo ATP in 2-PN, 4-cell and blastocyst stages in control group were $3.300 \times 10^{-12} \pm 0.46 \times 10^{-12}$, $2.99 \times 10^{-12} \pm 0.10 \times 10^{-12}$ and $3.16 \times 10^{-12} \pm 0.12 \times 10^{-12}$ mol and in vitrified-warmed embryos were $2.83 \times 10^{-12} \pm 0.24 \times 10^{-12}$, $3.01 \pm 0.42 \times 10^{-12}$ and $3.17 \times 10^{-12} \pm 0.12 \times 10^{-12}$ mol, respectively. The ATP concentration of embryos at 2-PN, 4-cell and blastocyst stages was not significantly different within each control and vitrified groups or between vitrified and non-vitrified samples ($P>0.05$).

The ATP content of the in vitro cultured mouse embryos. After *in vitro* culture of embryos at 2-PN and 4-cell stages, the ATP content of collected embryos in 4-cell and blastocysts stages was evaluated and compared subsequently. The ATP levels in 4-cell and blastocyst embryos, derived from cultured 2-PN embryos in control group, were $3.33 \times 10^{-12} \pm 0.30 \times 10^{-12}$ and $3.28 \times 10^{-12} \pm 0.61 \times 10^{-12}$ mol and in vitrified groups were $3.34 \times 10^{-12} \pm 0.23 \times 10^{-12}$ and $3.13 \times 10^{-12} \pm 0.13 \times 10^{-12}$ mol, respectively (Fig. 2A). The ATP levels of blastocyst embryos derived from cultured 4-cell embryos in control and vitrified groups were $3.07 \times 10^{-12} \pm 0.51 \times 10^{-12}$ and $2.87 \times 10^{-12} \pm 0.17 \times 10^{-12}$ mol, respectively (Fig. 2B). There were not

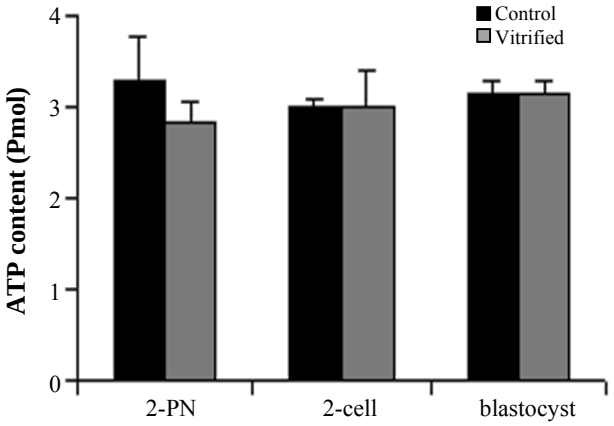

Fig. 1. The adenosine triphosphate (ATP) content of individually mouse embryos collected from in vivo condition at different developmental stages which subjected to vitrification in comparison with fresh (non-vitrified) group.

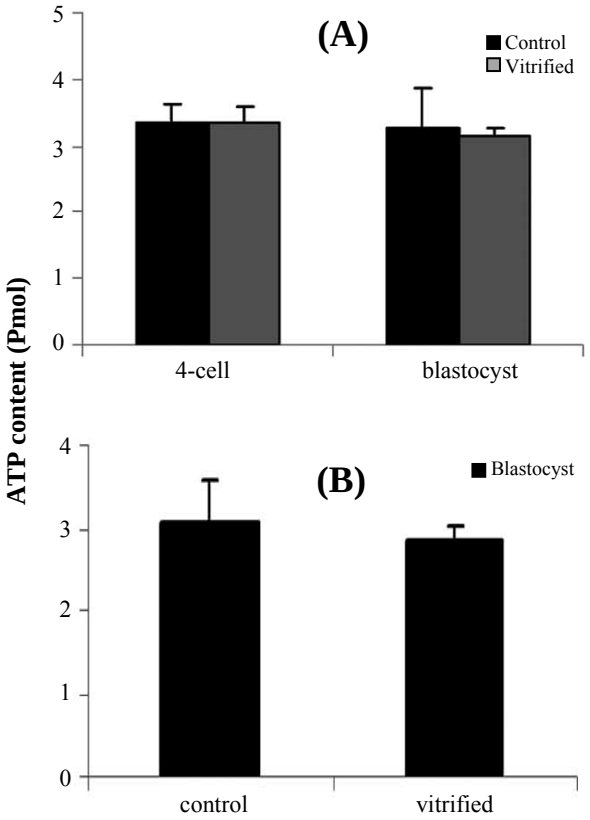

Fig 2. The comparison of adenosine triphosphate (ATP) content of individually mouse embryos derived from those cultured *in vitro* in vitrified and non-vitrified groups. **(A)** The level of ATP in 4-cell and blastocyst embryos derived from cultured 2-PN and 4-cell embryos respectively; **(B)** This level in mouse blastocyst derived from cultured 4-cell embryos in vitrified and non-vitrified groups.

significant differences in ATP concentrations of cultured embryos at different developmental stages in both vitrified and non-vitrified groups (P>0.05).

Distribution of mitochondria. Mitochondrial distribution of vitrified and non-vitrified embryos at 2-PN (Fig. 3) and 4-cell (Fig. 4) stages was observed under a fluorescent microscope using Mito-tracker green. Mitochondrial distribution in cytoplasm of 2-PN embryos in both vitrified and non-vitrified embryos showed a similar pattern. The mitochondria were diffused mainly within the cytoplasm homogenously and some mitochondria were localized around the pronucleus (Fig. 3). However, the pattern of mitochondrial distribution in 4-cell embryos was diffused within the cytoplasm of blastomers and there was not any sign of perinuclear aggregation. Some clampings or large aggregation of mitochondria within the vitrified samples was/were prominent (Fig. 4).

DISCUSSION

Present comparative study among embryos at different developmental stages demonstrated that cryotop method is suitable for vitrification of embryos at 2-PN, 4-cell and early blastocyst stages and the developmental stages of embryos did not affect the results of embryo vitrification. Therefore, the high rates of survival may relate to employed vitrification method with low toxic cryoprotectants in our study. Some of the previous reports have shown the similar results with our observations [8, 9]. However, usage of different vitrification techniques for cryopreservation of embryos in several mammalian species showed controversial results [4, 23].

In the present study, the effects of three factors on ATP content of mouse embryos were evaluated, including developmental stage of embryo, *in vitro* culture condition and vitrification and warming procedure. The ATP content of embryos at 2-PN, 4-cell and early blastocyst stages in both vitrified and non-vitrified groups was not significantly different. According to our observation, it is suggested that there is a balance between the ATP production and degradation during embryo development; therefore, the ATP content maintains constant.

In contrast, Sturmey and Leese [24] showed that the ATP content of porcine embryos at 4-cell stage was higher than 2-PN embryo and reached to maximum at early blastocyst stage. Rozell *et al.* [25] reported that ATP content of sheep embryos retained constant to 8-

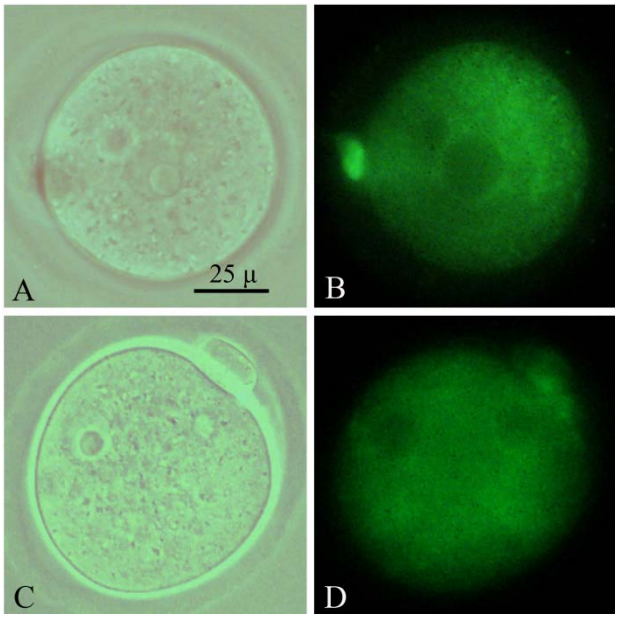

Fig. 3. The phase contrast micrograph of *in vivo* 2-PN mouse embryos in non-vitrified **(A)** and vitrified group **(C)**. Distribution of mitochondria stained by Mito-tracker green in non-vitrified **(B)** and vitrified group **(D)**.

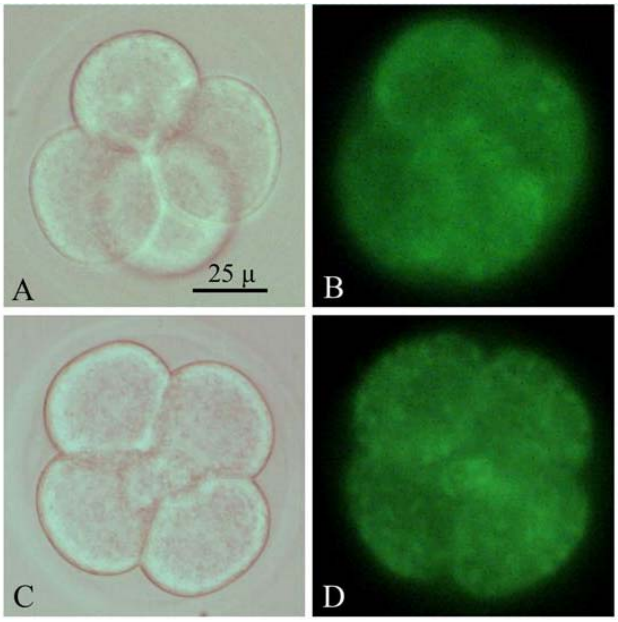

Fig. 4. The phase contrast micrograph of *in vivo* 4-cell mouse embryos in non-vitrified **(A)** and vitrified group **(C)**. Distribution of mitochondria stained by Mito-tracker green in non-vitrified **(B)** and vitrified group **(D)**.

cell stage, but decreased to morula stage and maintained low from morula to blastocyst stage. In another experimental study by Freistedt *et al*. [26], ATP level of cat embryos at 4-cell and 8-cell stages was more than 2-cell stage and reached to minimum at morula stage but increased at blastocyst stage. These controversies in ATP content may be due to the difference in species regarding to the structure of mitochondria within the preimplantation embryo or due to measuring technique of ATP concentration. ATP content of developing embryo may be related to energy production and consumption and mitochondrial number and maturation. Moreover, several cellular activities within the embryo require adequate energy production; therefore, ATP production can be increased during embryo development [27, 28].

All mitochondria of embryo inherited from the oocyte. The number of mitochondria was decreased in each cell division during the preimplantation stages; moreover, the structure of mitochondria has been matured during embryo development [29].

The present study is the first report on ATP assay of vitrified embryos at different developmental stages. According to our observation, the ATP content and cellular metabolism of embryos at different developmental stages was not affected by cryo-preservation. On the other hand, the cellular stress was not induced by vitrification procedure and the safety of this technique was shown.

In the present study, the ATP content of *in vitro* and *in vivo* produced embryos was similar. It indicated that the culture condition has not deleterious effect on embryo mitochondrial function and ATP content. In contrast, it was shown that before the culture condition more affect the cell metabolism and ATP levels [27, 28].

Another important factor in embryo development is mitochondrial distribution [11]. Mitochondrial distribution in 2-PN embryos in both vitrified and non-vitrified samples was similar and it was diffused within the cytoplasm. Also, there was some aggregation around the pronucleus as a ring. In contrast to our results, Zhao *et al*. [12] reported that mitochondrial distribution of embryos was altered after vitrification of mouse 2-PN embryos with open pulled straw method using ethylene glycol and DMSO. The differences between this report and our observations may be due to different vitrification methods. However in our study, the pattern of mitochondrial distribution in vitrified 4-cell embryos was different from non-vitrified embryos and this observation showed that the vitrification affected the mitochondrial distribution. The explanation for this alteration may be due to changes in embryo cytoskeleton, which mediated the distribution of mitochondria to different parts of the cytoplasm [30]. It was shown that during cooling and warming due to thermal shock, the cytoskeleton undergoes reversible or irreversible changes [31].

In conclusion, cryotop vitrification method using ethylene glycol and DMSO and *in vitro* culture condition did not affect the mouse embryo development and its ATP content at different developmental stages.

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