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Research Article

Investigation of the Vitrification Ability of Mouse Embryos with Quartz Capillary (QC) Straws

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ABSTRACT

The most frequent problems in current embryo freezing are formation of harmful ice crystals and toxicity of cryoprotectants. In slow freezing techniques although, the cryoprotectants can be used in a low concentration, ice crystals are formed and in rapid freezing process where ice crystals are avoided, this time toxicity of cryoprotectants deteriorates cells. New technological product quartz capillary straws have an ultra high temperature conductivity by which they avoid the necessity to use high concentrations of cryoprotectants and also prevent the formation of harmful ice crystals. The aim of this study was to investigate the use of quartz capillary straws for vitrification of mouse embryos. The quartz capillary straws have only been used to freeze murine embryonic stem cells and have not been tried in any multicellular embryo studies so far. In the study total 40 mouse embryos were frozen in six treatment groups with various concentration and combination of glycerol, ethylene glycol and trehalose. However, no live embryos were achieved after thawing and in vitro culture of embryos. In conclusion, high temperature conducting quartz capillary straws were observed to be inconvenient in freezing mouse blastocysts by vitrification with the type, concentration and combination of cryoprotectants used in this study.

Key Words: Mouse, embryo, vitrification, quartz capillary

ÖZET

FARE EMBRİYOLARININ VİTRİFİKASYONLA DONDURULMASINDA QUARTZ KAPİLLAR (QC) PAYETLERİN KULLANILABİLİRLİĞİNİN ARAŞTIRILMASI

Bilinen yöntemlerle yapılan embriyo dondurma çalışmalarında, zararlı buz kristallerinin oluşumu ve kriyoprotektanların toksisiteleri, karşılaşılan en büyük problemlerdir. Kademeli dondurma işleminde kriyoprtotektanlar daha düşük yoğunluklarda kullanılabilmesine karşın buz kristalleri oluşmakta; Vitrifikasyonda ise, buz kristallerinin oluşumu engellenirken bu defa kriyoprotektanların toksik etkilerinden kaynaklı hasarlar meydana gelmektedir. Yeni bir teknolojik ürün olan quartz kapillar payetler, çok yüksek ısı iletkenliği sağlaması sayesinde hem kriyoprotektanların yüksek yoğunlukta kullanılması zorunluluğunu ortadan kaldırmakta, hem de zararlı buz kristallerinin oluşumunu engellemektedir. Bu çalışmada, bu güne değin sadece sıçan embriyonik kök hücrelerinin dondurulmasında başarıyla kullanıldığı rapor edilen ancak, henüz herhangi bir multiselüler hücre çalışmasında denenmemiş olan Quartz Kapillar payetlerin, fare embriyolarının Vitrifikasyonla dondurulması üzerine etkinliğinin araştırılması amaçlandı. Altı farklı çalışma grubunda, gliserol, etilen glikol ve trehalose'un farklı yoğunluk ve

kombinasyonlarının denendiği ve toplamda 40 adet fare embriyosunun dondurulduğu çalışmada, çözdürme ve in vitro kültür sonrasında hiçbir embriyonun canlılığını sürdüremediği belirlendi. Sonuç olarak, çok yüksek ısı transferi sağlayan quartz kapillar payetlerin blastosist aşamasındaki fare embriyolarının vitrifikasyonla dondurulması için, kullanılan kriyoprotektif madde tip, oran ve kombinasyonlarının uygun olmadığı kanaatine varıldı.

Anahtar Kelimeler: Fare, embriyo, vitrifikasyon, quartz kapillar

Introduction

Towards the middle of the 20th century, with the use the glycerol successfully as a cryoprotectant in semen freezing (Polge et al., 1949), the studies on gamete freezing have been accelerated. The first successful freezing of mammalian embryos were reported in mice at 1972 (Whittingham et al., 1972) and in cattle at 1973 (Wilmut and Rowson, 1973). After this date, embryo transfer technology had pioneered great progresses in biotechnology area. In early years, fresh embryos were used in embryo transfer applications but, this has led to problems in field conditions. After cryopreservation of embryos successfully, embryos could be transferred to national and international fields and have become commercial dimension. The biggest problem of the fresh embryo transfer application is the necessity of the real time synchronization of both the donor and the recipients (Bucak and Tekin, 2007; Sağırkaya and Bağış, 2003). The viability rates of the freeze-thawed embryos are varied. The factors affecting the embryo viability can be listed as; animal species, the size and the developmental stage of embryos, freezing and thawing protocols, the permeability characteristics of the cells and the toxicity of the cryoprotectants. The embryo freezing methods can be defined as traditional slow freezing, rapid freezing and vitrification (Aksoy et al., 1999; Palasz ve Mapletoft 1996; Sağırkaya and Bağış, 2003). In slow-freezing method, expensive and complicated devices are needed, whereas in rapid freezing method at least two different cryoprotectants and high freezing rates are needed (Sağırkaya and Bağış, 2003). It is known that during the freezing processes, the embryos can be injured by increasing the intracellular cryoprotectant concentrations and by formation intracellular or intercellular ice crystals (Bucak and Tekin, 2007).

Rall and Fahy (1985) have recently developed a new freezing technique named Vitrification which no need for expensive and complicated devices and do not take place harmful ice crystals and create glassy vitreous state. In vitrification, the embryo holding straws plunged directly into liquid nitrogen for freezing, and this way saves time and economy. However, for a successful vitrification a rapid increase in the concentration of cryoprotectant is required (5-7M). This is approximately 2-3 times more than the concentrations used in the traditional slow freezing technique. In vitrification, 0.25 ml plastic straws are plunged directly into liquid nitrogen and the highest freezing rate provided is approximately 2500 °C/min. In addition, open pulled straws, electron microscopic grid and cryoloop can be used for this purpose too. The heat transfer rate provided by these materials is approximately 50000 °C / min or less and the concentration of the cryoprotectants used in this techniques should be so high (He et al., 2008; Jain and Paulson, 2006). Some researchers have reported that they could freeze murine embryonic stem cells successfully by using very low concentration (2 M) of cryoprotectant in a newly developed quartz capillary straws (QC) providing ultra high thermal conductivity (He et al., 2008). The superiority of the quartz capillary straws is providing ultra high thermal conductivity (> 100000 °C / min) by the aid of their wall thickness (0.01mm) and hence eliminating the necessity to use high concentration of cryoprotectants (Risco et al., 2007). Thus, QC straws allow using the cryoprotectant less toxic, because of useing in small quantities and lower concentrations. This rapid vitrification method is also called ultra rapid vitrification. In this method the volume of vitrification solution minimizes and thus the cooling rate increases. The minimization of the

vitrification solution provided not only maximizes the freeze-thaw rates but also reduces the production of the harmful ice crystals (Berejnov et al., 2006).

The quartz capillary straws have only been used to freeze murine embryonic stem cells, and have not been tried in any multicellular embryo studies so far. Hence, the aim of this study was to investigate the use of quartz capillary straws for freezing mouse embryos by vitrification.

Materials and methods

Balb/C strain 8-10 weeks old, 10 female and five male mice were the animal materials of the study. The mice were kept under 12/12 light/darkness cycle, 50% humidity and 22-24^{o}C warm conditions. The mice were fed with pellets including 20% protein and water ad libitum. For estrus synchronization and superovulation, 5 I.U. PMSG and 48 h later 5 I.U. hCG were given to the females intraperitonally. Then the females were kept with the males by one to one in cages for mating and were checked next morning for presence of vaginal plaque. Females with the vaginal plaque were accepted as pregnant and were killed by cervical dislocation after four days. The blastocyst stage embryos collected from uterus with M 2 flushing medium (Evecen et al., 2004).

Six different cryoprotectant combinations (six trial groups) were used in this study. All chemicals were provided from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise indicated.

The cryoprotectant solution names, concentrations, equilibration times and numbers of embryos used in different six trial groups as follows:

Trial Group 1: (5 embryos)

Cryoprotectants	**Equilibration Time (min)**
1.5 M Glycerol (G 2025) + 0.3 M Trehalose (T 0167)	10
1.5 M Glycerol +1.5 M Ethylene Glycol (S44669-118) +0.5 M Trehalose	1.5
2 M Glycerol +2 M Ethylene Glycol + 0.5 M Trehalose	1.5

Trial Group 2: (8 embryos)

Cryoprotectants	**Equilibration Time (min)**
1.5 M Glycerol + 0.3 M Trehalose	10
1 M Glycerol +1 M Ethylene Glycol +0.5 M Trehalose	5
1.5 M Glycerol +1.5 M Ethylene Glycol + 0.5 M Trehalose	5

Trial Group 3: (7 embryos)

Cryoprotectants	**Equilibration Time (min)**
1.5 M Glycerol + 0.3 M Trehalose	10
2 M Glycerol +2 M Ethylene Glycol +0.5 M Trehalose	3
3 M Glycerol +3 M Ethylene Glycol + 0.5 M Trehalose	30 seconds.

Trial Group 4: (8 embryos)

Cryoprotectants	**Equilibration Time (min)**
1.5 M Glycerol	10
2 M Glycerol + 2 M Ethylene Glycol	1

Trial Group 5: (6 embryos)

Cryoprotectants	**Equilibration Time (min)**
1.5 M Glycerol	5
2 M Glycerol + 2 M Ethylene Glycol	3

Trial Group 6: (6 embryos)

Cryoprotectants	**Equilibration Time (min)**
1 M Glycerol	14
1.5 M Glycerol + 1.5 M Ethylene Glycol	5

In the study commercial Quartz Capillary straws (QC) (The Charles Supper Company, Inc.) with 0.3 mm internal diameter, and 0.01 mm wall thickness were used. Embryos were loaded to the QC straws an embryo to one straw basis with the minimum volume (5-10 µl) of vitrification media. The embryos transferred to minidrops of cryopreotectants and then, the QC straws contacted to the embryo included minidrops. The embryo and little volume of cryoprotectants were passed to the QC straws immediately by capillarity. Immediately afterwards, the straws were frozen by directly plunging into liquid nitrogen. The next day, the straws were thawed in different concentrations of sucrose by three steps. The embryos were equilibrated for five minutes in each concentration (1, 0.5 and 0.25 M respectively) of sucrose. In this process the heat of the sucrose solution was adjusted to 37°C. Then the embryos were washed three times in PBS+FCS solution for removal of all cryoprotectants and sucrose remains and transferred to the culture media (KSOM) in a humidified atmosphere of 5% CO_2 in air and 37°C incubator conditions for 24 h. (Erbach et al., 1994). Next day, the viability of the embryos was checked and the results were recorded.

Results and Discussion

At the end of the study, it is observed that the freeze-thawed embryos which were in six treatment groups (40 embryos) were degenerated.

The cryoptotectants are used for preventing the cold shock, intracellular ice crystal formation and membrane destabilization of the cells during freezing and thawing processes (Bucak and Tekin, 2007). The cell membrane is permeable to water more than all the cryoprotectants and when cryoprotectant added to the medium the cell's first reaction would be to shrink. Dehydration slows down when the cryoprotectants enter the cells and if the cells are exposed a long time to the cryoprotectants return to their original shape (Sağırkaya and Bağış, 2003; Wowk, 2010). In vitrification, the ice crystal formation is prevented and by the temperature falls down the cryoprotectants passes a phase of viscous and glassy vitreous state (Wowk, 2010). According to two-factor hypothesis of Mazur (1990); if the cooling process proceeds for a longer time, the cells will be damaged due to exposure to hyper osmotic environment for a long time and if the process lasts shorter, the cells immediately lose their energy and dehydration rate slows. Therefore, a large amount of water remains in the cell and the cell damages due to formation of ice crystals. The permeability of the cell membranes against the cryoprotectants is as aid in choosing the most suitable freezing technique. The permeability changes of the cell membranes are related to the type of cryoprotectant as well as the developmental stage of the cell and strain of the animal (Bucak and Tekin, 2007; Mazur, 1990; Sağırkaya and Bağış, 2003). When the embryos start division the blastomere volume decreases and the surface area/volume ratio increases. So, the interaction of blastomeres and their water permeability changes. At the advanced developmental stages of embryos, the large membrane surface creates risk and membrane permeability increases with the divisions. The membrane permeability of the embryo varies according to developmental stage and quality of the cells (Berejnov et al., 2006; Wowk, 2010). Although the high temperature conducting quartz capillary straws have been used successfully to freeze the murine embryonic stem cells, they are observed to be inconvenient in freezing mouse blastocysts by vitrification with the type, concentration and combination of cryoprotectants used in this study. It seems that the ultra high thermal conductivity (> 100000 °C / min) of the straws, probably causes lower speed of cell dehydration of the surface area /volume ratio increased and differentiated characteristics of the membrane water permeability blastocyst stage embryo. Thus, the intracellular fluid forms crystals and damages the cells.

In conclusion, high temperature conducting quartz capillary straws were observed to be inconvenient in freezing mouse

blastocysts by vitrification with the type, concentration and combination of cryoprotectants used in this study. More studies in this field by using various concentration and combination of cryoprotectants would be beneficial aspects.

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REFERENCES

Aksoy, M., Takahashi, Y., Hishinuma, M., Elsbeikh, A.S., Tanaka, A., Kanagawa, H., 1999. Influences of retrieval stages and glutathione addition on post-thaw viability of quick frozen Mouse Morula during in vitro culture. Theriogenology 51, 681-687.

Berejnov, V., Husseini, N.S., Alsaied, O.A., Thorne, R.E., 2006. Effects of cryoprotectant concentration and cooling rate on vitrification of aqueous solutions. Journal of Applied Crystallography 39, 244-251.

Bucak, M.N., Tekin, N., 2007. Kryoprotektanlar ve gamet hücrelerinin dondurulmasında kryoprotektif etki. Ankara Üniversitesi Veteriner Fakültesi Dergisi, 54, 67-72.

Erbach, G.T., Lawitts, S.A., Papaioannou, V.E., Biggers, J.D., 1994. Differential growth of the mouse preimplantation embryo in chemically defined media. Biology of Reproduction 50, 1027-1033.

Evecen, M., Pabuccuoğlu, S., Alkan, S, İleri, İ.K., 2004. The effects of various BSA levels on development in in vitro culture of mouse embryos. Turkish Journal of Veterinary and Animal Sciences 28, 337-342.

He, X., Eric, Y.H.P., Fowler, A., Martin, L.Y., Toner, M., 2008. Vitrification by ultra-fast cooling at a low concentration of cryoprotectants in a quartz micro-capillar: A study using murine embryonic stem cells. Cryobiology 56 (3), 223-232.

Jain, J.K., Paulson, R.J., 2006. Oocyte cryopreservation. Fertility and Sterility 86, 1037-1046.

Mazur, P., 1990. Equilibrium, quasi-equilibrium, and nonequilibrium freezing of mammalian embryos. Cell Biophysics 14, 53-92.

Palasz, A., T, Mapletoft, R.J., 1996. Cryopreservation of mammalian embryos and oocytes: Recent advances. Biotechnology Advence 14, 127-149.

Polge, C., Smith, A.U., Parkes, A.S., 1949. Revival of spermatozoa after vitrification and dehydration at low temperatures. Nature 164, 666-667.

Rall, W.F., Fahy, G.M., 1985. Ice-free cryopreservation of mouse embryos. Nature 313, 573-574.

Risco, R., Elmoazzen, H., Doughty, M., He, X., Toner, M., 2007. Thermal performance of quartz capillaries for vitrification. Cryobiology 55, 222-229.

Sağırkaya, H., Bağış, H., 2003. Memeli embriyolarının kriyoprezervasyonu. Uludağ Üniversitesi Veteriner Fakültesi Dergisi 22 (1-2-3), 127-135.

Whittingham, D.G., Leibo, S.P., Mazur, P., 1972. Survival of mouse embryos frozen to –196 and – 269°C. Science 178, 411-414.

Wilmut, I., Rowson, L., 1973. The successful low temperature preservation of mouse and cow embryos. Journal Reproduction Fertility 33, 352-353.

Wowk, B., 2010. Thermodynamic aspects of vitrification. Cryobiology 60, 11-22.