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Effects of blastocyst artificial collapse prior to vitrification on hatching and survival rates and the expression of klf4 gene in mouse embryos

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Abstract

Although the rate of blastocysts implantation of embryos is higher than previous stages but their survival rate is lower than them, which could be attributed to the completely filled blastocoel cavity with liquid and increased possibility of the formation of ice crystals. This liquid could prevent the penetration of cryoprotecting materials into the embryos. In this study, we reduced the volume of blastocoel before vitrification and compared survival rate and quality of *in vitro* embryos through klf4 gene expression with control group. *In vitro* mouse blastocysts were divided into three groups. In group 1, the blastocoel volume of blastocysts were reduced before vitrification and warming. In group 2, blastocysts were just vitrified and warmed and the blastocysts of group 3 (control group) were not undergone any specific treatment and were not vitrified. The expression of klf4 gene was assessed using real-time PCR technique. Data were statistically analyzed using one-way ANOVA and Duncan's post hoc tests. Our results showed that blastocoel volume reduction before vitrification significantly increased the hatching rate of the blastocysts from the zona pellucida and klf4 gene expression compared to vitrified group. Blastocoel volume reduction before vitrification could be used as an efficient method for improving the rate of *in vitro* fertilization.

اثرات کاهش حجم مصنوعی بلاستوسیست پیش از انجماد شیشه‌ای بر روی میزان بقاء و تفریخ و بیان ژن klf4 در رویان موش

چکیده

گرچه میزان لانه گزینی بلاستوسیست ها در مقایسه با مراحل پیشین رویانی بیشتر است، اما میزان زنده مانی آنها پایین‌تر می‌باشد که می تواند ناشی از پر بودن کامل حفره بلاستوسل از مایع و افزایش احتمال تشکیل بلورهای یخ باشد . این مایع می تواند از نفوذ مواد محافظت کننده در برابر سرما به درون رویان ها جلوگیری کند. در این مطالعه، حجم بلاستوسل را قبل از انجماد شیشه‌ای کاهش و میزان زنده مانی و کیفیت رویان های آزمایشگاهی را از طریق بیان ژن klf4 با گروه شاهد مورد مقایسه قرار دادیم. بلاستوسیستهای آزمایشگاهی موش به سه گروه تقسیم شدند. در گروه ۱، حجم بلاستوسل بلاستوسیستها قبل از انجماد و ذوب شدن، کاهش یافت. در گروه ۲، بلاستوسیستها تنها منجمد و ذوب شدند و بلاستوسیستهای گروه ۳ (گروه شاهد) متحمل هیچ نوع تیماری نشدند و ذوب نگشتند . بیان ژن klf4 با روش سنجش واکنش زنجیره ای پلیمراز در زمان واقعی ارزیابی شد. داده ها توسط روش ANOVA یک طرفه و آزمون تعقیبی دانکن مورد ارزیابی آماری قرار گرفتند. نتایج ما نشان داد که کاهش حجم بلاستوسل قبل از انجماد شیشه ای به طور معنی داری میزان خروج بلاستوسیستها از ناحیه شفاف و بیان ژن klf4 را در مقایسه با گروه انجماد شیشه‌ای افزایش داد. کاهش حجم بلاستوسل قبل از انجماد شیشه‌ای می تواند به عنوان روشی کارا جهت بهبود میزان لقاح آزمایشگاهی مورد استفاده قرار گیرد.

واژه های کلیدی: انجماد شیشه‌ای، بلاستوسیست، ژن klf4، کاهش حجم مصنوعی، لقاح آزمایشگاهی

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Introduction

In vitro fertilization (IVF) is the most common assisted reproduction technique. Embryo transfer to the uterus is possible at all stages but the best stage is the blastocyst regarding to its compatibility with uterus environment. The transfer of embryos at the blastocyst stage reduces the risk of multiple pregnancies. Recently, increasing attention is being paid to the optimization of embryo culture conditions and transfer at the blastocyst stage in order to increase the possibility of implantation and successful impregnation rate due to the transfer of embryos from IVF. In IVF treatment, usually 2-3 embryos at 4-8 cells stages are transferred to the uterus of the recipient which has a lower pregnancy rate compared to transfer at the blastocyst stage. Using consecutive culture mediums and development of the embryos up to the blastocyst stage *in vitro*, we can transfer the embryo at this stage. The transfer of a blastocyst at this stage improves the pregnancy rate as well as reduces the rate of multiple pregnancy leading to better mother and child health because of the higher growth potential of the embryo at this stage.[1-3]

It has been reported that embryos that cannot maintain their biological capabilities until the blastocyst stage most probably have a small number of abnormal chromosomes. Therefore, embryo culture up to the blastocyst stage could act as a selective measure for preventing genetic disorders and maintaining healthy embryos.[4] Another advantage of transfer at blastocyst stage is better consistency of embryos at this stage with uterine wall.[5-7]

One of the limiting factors in blastocyst transfer is freezing and storage of embryos in this stage. When one embryo is transferred to the uterus, the remaining ones should be frozen and stored to be used in other treatment cycles or donation to other infertile couples. Embryos are stored using two methods: slow freezing and vitrification. The first method is based on using lower concentrations of cold-protecting materials and gradual reduction of temperature. This method is accompanied by a reduction in the rate of cell survival after thawing because of the higher possibility of ice crystals creation and lower humidity rate. In the second method known as vitrification, the embryos are frozen without formation of ice crystals because of using a combination of cold-protecting materials in high concentrations and direct embryo contact with the lowest volume of freezing solution with liquid nitrogen.[6]

Freezing can be done in all stages of development from two cells to blastocysts. However, the challenge is attaining a high survival rate and increasing successful implantation after warming and transfer. Freezing embryos have higher survival rates compared to blastocysts in the first stages of cleavage, but they cannot be morphologically predictable in subsequent developmental stages and they increase the rate of pregnancy failure and this is why we tend towards freezing of blastocysts. The main problem in blastocysts freezing is the existence of liquid filled blastocoel cavity which limits their access to inner cells as well as the transfer speed of cold-protecting materials and increases the potential of ice crystals formation. To overcome this problem, higher concentrations of cold-protecting materials should be used or duration of embryo exposure to these materials should be longer, both of which can harm the embryo.[8]

Krüppel-like factor (klf4) gene is one of pluripotency specific genes. On the other hand, klf4 is essential for blastocyst development and normal self-renewal of embryonic stem cells.[9] Direct reprogramming of somatic cells into embryonic like stem cells by over-expressing of only four transcription factors (Oct4, sox2, klf4 and c-Myc) has great potential for regenerative medicines, removing the ethical issues of the use of embryonic stem cells and rejection of problems of using non-autologous cells.[10] However, reprogramming efficiency rate is low and risks of introduction of viral genetic materials are only partly researched.[10] Recently, over-expressing of klf4 and Oct4 alone has been proposed to reduce the number of virally expressed transcription factors at reprogramming cells into induced pluripotent stem cells.10 This show the importance of klf4 and Oct4 genes in pluripotency of cells and early embryos.

The purpose of this study was to investigate the effects of artificial collapse (AC) before vitrification on survival rate and expression of klf4 pluripotent gene in the vitrified-warmed mouse blastocysts.

Materials and Methods

The Institutional Animal Care and Use Committee of the National Institute of Genetic Engineering and Bio-technology of Iran approved all experiments in this study (IR.NIJEB.FC 01.11.2015-1).

In vivo-produced blastocysts were pooled together and then randomly divided into three groups: The first group were reducing the blastocoel volume of blastocysts before vitrified. The second group of blastocysts was vitrified and warmed. The third group, as a control for culture conditions. Survival and hatching rates were investigated within 12 hr of warming or incubation.

Animals and oocyte collection. The 10-12 weeks old mice were used in the present experimental study. Male mice were kept in 12/12 hr light/dark cycles at 18 ˚C. *In vitro* mouse blastocysts were divided into three groups. In group 1, the blastocoel volume of blastocysts were reducing before vitrified and warmed, in group 2, blastocysts were just vitrified and warmed and the blastocysts of group 3 (control group) were not undergone any specific treatment and were not vitrified. On the first

day, 8.00 IU pregnant mare serum gonadotropin (Intervet, Boxmeer, The Netherlands) was intraperitoneally injected to female Naval Medical Research Institute (NMRI) mice (6-8 weeks old) to stimulate ovulation. After 48 hr, for releasing the eggs, 7.00 IU human chorionic gonadotropin (Intervet) was intraperitoneally injected. At least 4 hr before egg extraction, collection and washing, modified Krebs-Ringer solution with HEPES- buffered M2 medium (Sigma, St. Louis, USA) environment with sodium pyruvateg (Sigma) and bovine serum albumin (Sigma) was placed in a Petri dish and put in the incubator after being covered with oil.[11] The male mice were sacrificed by cervical dislocation and placed in the supine position and the abdominal cavity was exposed. The sperms were extracted from the cauda epididymis and placed in the carrying capacity environment for 1 hr.

After incising the abdomen and exposure of the uterus and ovaries, the oviducts were cut with scissors and transferred to the M2 drops. Under the microscope, their ampullary region was punctured with a needle and oocyte-cumulus masses were released. These masses were stored in the incubator after being washed.

The IVF procedure, embryo culture and blastocysts puncture. For IVF, M2 environment plus sodium was used. The 50 mL drops from human tubal fluid (HTF; SAGE, Trumbull, USA).[12] (containing 1.00% human cord serum failed to support blastocyst hatching) culture were put in the Petri dish and placed in the incubator after being covered with sterile mineral oil. Ten to fifteen eggs with cumulus which appeared normal were placed in each HTF drop and sperms with a concentration of one million per mL were added to each drop.

For embryo culture, medium containing sodium pyruvate was used. After pouring 400 mL of potassium synthetic oviduct medium (KSOM; Millipore, Billerica, USA) solution in each well of the plate, the four T holes of the surface were covered with the same volume of sterile mineral oil. Fertilized oocytes were then washed and transferred to four section dishes.[13] For puncturing the blastocysts, we used a micromanipulator microscope. Grown blastocysts were separately placed inside each M2 drop with a Pasteur pipette. Then, under the microscope, the blastocyst was punctured with an injection needle and the holding needle installed on the device, so that the cells of inner mass were not in line with the entrance and exit of the needle inside the embryo. Then, the needle was slowly inserted into the embryo between the trophectoderm cells.

After several seconds, the needle was slowly taken out while the embryo was still fixed using negative pressure by holding needle.

Vitrification. For vitrification, Tissue Culture Medium 199 (TCM-199; Gibco, Inchinnan, Scotland) was used as base environment for washing. Then, base environment contain 7.50% dimethyl sulfoxide (DMSO; Sigma) and 7.50% ethylene glycol (Sigma) was used. Freezing solution contains base environment plus 15.00% dimethyl sulfoxide and 15.00% ethylene glycol and 0.50 M sucrose (Sigma). The blastocysts were loaded on cryotype with the least freezing environment volume and submerged in nitrogen. For warming the embryos, base environment plus 1 M sucrose as a main environment was used. All vitrification and warming solutions were put in room temperature 1 hr before the experiment. The warmed embryos were transferred to KSOM environment and assessed after 12 hr. The survival rate was assessed through further expansion of embryos and exit rate of blastocysts from zona pellucida using a stereo microscope. The penetration ratio of 280A/260A was used as a criterion for lack of protein contamination.

Real time PCR. The RNA was extracted from sample cells using Trizol RNeasy Plus Micro Kit® (Qiagen, Valencia, USA), The cDNA was synthesized using cDNA synthesis kit (AccuPower Rocketscript RT Premix; Bioneer, Taejeon, Korea) based on the manufacturer's instruction. Primers were designed using NCBI website and synthesized by Bioneer. Using SYBR Green master mix (Bioneer), PCR reactions were performed by ABI StepOne (Applied Biosystems, Foster City, USA). The QRT-PCR program was performed with a melting cycle for 5 min at 95 ˚C and then, 10 secs at 95 ˚C by 40 cycles of melting, 15 secs at 60 ˚C (annealing) and 30 secs at 72 ˚C extension (Table 1). The sequences klf4 and GAPDH are as follow: Forward: 5′-AAGAACAGCCACCCACACTTG-3´and Reverse: 5´-GTAAGGTTTCTCGCCTGTGTGA-3´, Forward: 5′- ATGGG GAAGGTGAAGGTCG-3´ and Reverse: 5´ -GGGGTCATTGATG GCAACAATA- 3´, respectively.

Statistical analysis. Data were analyzed using one-way ANOVA and Duncan's post hoc tests and value of $p < 0.05$ was considered statistically significant.

Results

The results of effects of blastocyst AC prior to vitrification on hatching and survival rates of 656 mouse embryos have been shown in Table 2. There was an

Table 1. The real time PCR program features for klf4 gene.

Stage	Phase	Cycle number	Temperature (˚C)	Time (sec)
Holding	1	1	95	120
Cycling	Denaturation	40	95	10
	Annealing		55	30
Melt curve	1	1	95	30
	2	1	55	60

Table 2. Effect of *in vitro* blastocyst artificial collapse (AC) before vitrification on quality of warmed embryos in mice.

Groups	No. of embryos examined	No. of surviving embryos (%)	No. of hatched embryos (%)
Control	262	100	67.73 ± 10.40[a]
Artificial collapse / Vitrification	194	91.76 ± 2.68[a]	58.70 ± 6.55[b]
Vitrification without AC	200	95.54 ± 5.12	44.40 ± 10.65

[ab] Values with different superscripts within a column denote a significant difference compared to the other groups ($p < 0.05$).

insignificant reduction in survival rate (%) of vitrified-warmed blastocysts group (95.54 ± 5.12) compared to control group (100%). Also, there was a significant reduction in hatching rate (%) of this group (44.40 ± 10.65) compared to control group (67.73 ± 10.40). After AC, survival and hatching rates were 91.76 ± 2.68 and 58.70 ± 6.55, respectively ($p < 0.05$). Also, increased expression of klf4 gene was observed in treated groups compared to control group (Fig. 1).

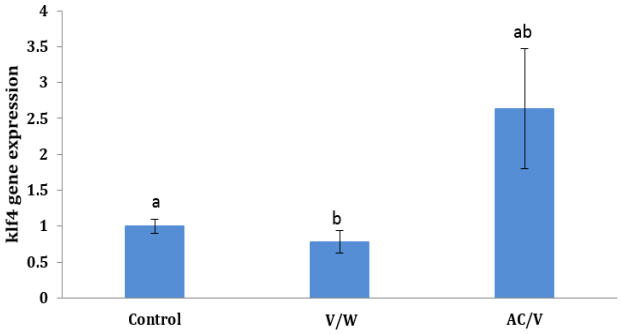

Fig. 1. The klf4 gene expression analysis in artificial collapse/vitrified (AC/V) group compared to vitrified/warmed (V/W) and control groups by real time PCR. Data were pooled from three replicates. [ab] Values with different superscripts within a column denote a significant difference compared to the other groups ($p < 0.05$).

Discussion

Cryopreservation is possible for all stages of pre-implantation embryos. It has been reported that survival rate of blastocyst is comparably lower than other stages.[14] There is a high volume of fluid in blastocoel cavity that can be a good ground for ice crystals formation, resulting in cellular damages. Here, the effects of AC and reduction of fluid volume in blastocoel cavity before vitrification process on the survival rate, hatching level and quality of blastocysts were assessed by estimating the expression levels of Klf4. The reduction of survival rates of embryos under vitrification and warming is due to formation of intra- and extracellular ice crystals. Although vitrification can prevent the formation of such crystals, using cryo-protectants at high doses will damage the embryo, which therefore decreases the survival rate of it.[9] It has been shown that vitrification has damaging effects on the inner and outer cell masses.[15] It has been suggested that vitrification and warming increase necrosis as well as apoptosis in embryonic cells.[16] The increase in survival rates of embryos following AC might be due to hole formation in zona pellucida and faster penetration of cryoprotectants into the embryo.[17]

Researchers have observed significant improvement in artificial collapsed blastocysts compared to control group.[18] Others have reported similar results obtained through puncturing blastocyst using needles.[19] In our study, a decrease in the survival rate of AC embryos compared to that of the vitrified group was observed that could be due to hardness of zona pellucida in IVF embryos. In this study, the embryos exit rate from zona pellucida was increased significantly compared to vitrified group. It could be due to lack of blastocysts energy for returning to the initial state after thawing. The initial state required to consume energy. Our results showed that in AC group the removal of zona pellucida increased compared to vitrified group indicating improvement of zona pellucida removal by AC. The removal of blastocyst from zona in IVF is done by multiple holes formation in the membrane, which is different from removal of blastocyst from zona in uterus. The removal of blastocyst from zona in IVF and *in vivo* is fundamentally different. The scientists did not find any difference in the survival rates of non-vitrified embryos and vitrified-warmed embryos and concluded that healthy zona is a negative factor in embryo vitrification.

We also examined the effects of AC on specific outcome parameters such as hatching rate. It has been reported that increasing of hatching rate by AC may prevent any possibility of long-term damages to the fetus.[20] Our results showed that vitrification with AC exhibits a higher rate of hatching. It has been reported that laser technique appears to be effective on hatching rate of blastocysts.[20] It seems that despite little overt indication of damage, AC can reduce a higher proportion of dead cells in expanding late blastocysts vitrified without AC. Indeed, compared to early blastocysts, hatching blastocysts were far more vulnerable during vitrification. The reduction of internal fluid volume by AC through mechanical puncture or laser pulse minimized cell damages during vitrification-warming.[20] Our data support the theory that larger blastocoels may interfere with the vitrification process, allowing ice crystals formation and subsequent cellular damages. It has been suggested that application of AC in expanded blastocysts could potentially enhance vitrification outcomes through increasing hatching rate of blastocysts.[21]

It has been shown that AC can reduce the damages in the genome of fetus during freezing and thawing. Our

results showed that klf4 gene expression in freezing group has no significant difference with control group, while it showed significant increase in AC group. Differentiation of each embryo cell to inner cell mass (ICM) and outer cell mass (OCM) is under genetic control. A reduction in the number of cells and ICM cells nuclei size increase in the IVF group compared to *in vivo* group has been reported previously.[18] Indeed, it has been shown that the survival rate of ICM cells in IVF embryos is lower than OCM cells. In IVF this is usually associated with decreased number of ICM cells.[22] This can be attributed to the effect of stresses caused by environmental and osmotic conditions changes and ectopic pH. Therefore, there will be a change in ratio of ICM cells compared to OCM cells and gene expression change. No report has been registered based on molecular analysis of AC effect on gene expression rate so far. It is concluded that in AC group, klf4 gene expression shows a significant increase. The reason could be a response to the loss of damaged blastomeres to maintain the level of gene expression. Considering the key role of klf4 gene in growth and OCM cell proliferation, the change in this gene expression has abnormal complications on the embryonic cells.

In conclusion, there was a significant reduction in survival and hatching rates of vitrified-warmed blastocysts after AC compared to vitrified-warmed blastocysts group. In addition, there were significant changes in expression of klf4 gene in all groups. It can be concluded that AC could be a simple and effective way for successful blastocyst vitrification.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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