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Effect of Vitrification of Zygotes in Nylon Mesh on the Differential Expression of Apoptotic Genes in Mouse Embryos

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Abstract: Vitrification offers a rapid and simple alternative to cryopreservation by slow cooling methods. The objective was to develop an improved method to cryopreserve large number of cumulus intact mouse zygotes by vitrification using nylon mesh. Vitrified mouse embryos were assayed for the expression of apoptosis related genes at various stages of pre-implantation development. For vitrificaiton, zygotes were transferred to the cryoprotectant solution A, composed of 10% (v/v) ethylene glycol (EG), 4.5% (w/v) Ficoll-70 and 0.075 M sucrose in modified Dulbecco's Phosphate Buffered Saline (PBS) for 7 min followed by solution B consisting of 20% (v/v) EG, 9.0% (w/v) Ficoll-70 and 0.15 M sucrose in PBS for 2 min and finally in solution C, composed of 40% (v/v) EG, 18% (w/v) Ficoll-70 and 0.3 M sucrose in PBS for 1 min. After equilibration, 30 to 40 cumulus enclosed zygotes were loaded onto the nylon mesh, immediately transferred to 2 ml cryovials and directly plunged into Liquid N_2 within 40 to 60 sec. Post warming of vitrified zygotes resulted in 81.7% morphological survival. There were high rates of blastocyst development (59.9%), but the development was lower than controls (66.2%; $P < 0.05$). Still, there was no difference in the hatching rates between groups. Both Fas L and Fas mRNA were detected at the 4-cell and morula stages of development, suggesting that Fas-Fas L system might be operational in the early embryos. The level of expression of Bax mRNA tended to increase, while expression of survivin mRNA was not different for 2- and 4-cell embryos. Fragmented embryos showed an increase in Bax mRNA levels, while survivin mRNA level was reduced. Our data demonstrated that vitrification of cumulus intact zygotes using nylon mesh resulted in successful survival and development to the blastocyst stage.

Key words: Nylon mesh · Gene expression · Vitrificaiton · Zygote · Mouse

INTRODUCTION

The mouse embryo was the first mammalian embryo to be frozen successfully [1] and the first successful vitrification of mammalian embryos was reported [2] in the mouse. Since this initial effort, embryos of many mammalian and a few non-mammalian species were successfully cryopreserved [3]. Cryopreservation of mouse embryos is a cost-effective approach for the maintenance of scientifically important stocks, strains and lines [4]. The use of cryopreserved mouse zygotes would provide a more efficient approach to the production of transgenic mice using pronuclear injection by reducing or eliminating the need for on-site maintenance of animals serving as embryo donors and thereby, reduce the costs associated with animal housing [5, 6]. Furthermore, mouse embryo banking is an important technical supportive tool for genome preservation purposes. The efficiency of embryo banking for mouse models of human disease and normal biological processes depends on the ease of obtaining embryos.

Nevertheless, the developmental stage at which cryopreservation is performed varies among different species. This is determined by the practicality of recovery techniques, culture methods and by embryo survival rates after cryopreservation [7]. Mouse embryos can be readily recovered from the oviductal ampulla as zygotes, but are routinely cryopreserved at the 8-cell stage because higher survival rates were obtained at this stage [8]. Although cryopreservation of zygotes is usually more difficult than

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later embryonic stages, these embryos were successfully cryopreserved either by conventional slow freezing or vitrification methods [5, 6, 9]. But, the time consuming and laborious process of slow cooling has made vitrification an attractive alternative for freezing embryos. New methods based on the immersion of very small amounts of solutions into liquid nitrogen were developed to increase speed (>10000°C /min) of freezing and warming [10-12]. A simple and inexpensive vitrification method utilizing nylon mesh was described recently to vitrify large numbers of bovine germinal vesicle oocytes with high rates of survival [13]. However, the effectiveness of nylon mesh vitrification methods for cryopreservation of mouse embryos has not been tested. The pore size of the nylon mesh is ~60 μm, making it questionable for use as a support structure for cryopreservation on mouse embryos.

Regardless of the method of freezing employed, the freezing and thawing processes decrease embryo viability, an effect attributed to physical and chemical damage induced during cryopreservation [14, 15], triggering a cell stress response that can activate the apoptotic cascade or lead to necrosis [16]. During development of mouse embryos, different anti- and pro-apoptotic genes are expressed [17, 18]. Apoptosis, also known as programmed cell death, is a coordinated event dependant upon the actions and interactions of a number of gene products that either suppress or activate the process of cellular self-destruction [19]. Transcript analysis of apoptosis related and/or stress related genes is useful for examining the quality variation of embryos [20]. Yet, there is lack of information about vitrification induced changes in apoptotic genes during early development in mouse embryos.

The fate of the cell is determined by the ability of the cell death suppressors to sequester and neutralize the actions of the cell death inducers. Hence, the relative concentration of pro- and anti-apoptotic genes determines whether a cell lives or dies [21]. *Fas L* (CD95L), a cell surface molecule belonging to the tumor necrosis factor family, binds to its receptor *Fas* (CD95), inducing apoptosis of *Fas*-bearing cells. *Bax*, a proapoptotic mRNA transcript, appears consistently expressed throughout preimplantation development, with only a slight increase in accumulation of the protein in low quality embryos [22]. An increased transcription of apoptosis related genes, Fas and Survivin, reducing the developmental capacity of frozen thawed bovine blastocysts was reported [23]. Survivin is a mammalian protein that acts as an inhibitor of apoptosis proteins.

The overall objective of this study was to evaluate a procedure to cryopreserve large numbers of mouse pronuclear stage embryos successfully for later use. Specific objectives were: 1) step-wise vitrification of large numbers of cumulus intact mouse zygotes using nylon mesh and determining their developmental capacity compared to fresh embryos; and 2) establishment of a link between stage-dependant development and the differences in the expression of Fas L, Fas, Bax, Bcl2 and survivin.

MATERIALS AND METHODS

Embryo collection: Female ICR mice were superovulated by an intraperitoneal injection of 5 IU eCG (Sigma Chemical Co., St. Louis, MO, USA) followed 48 h later by 5 IU hCG (Sigma). Females were placed with males immediately after hCG administration and presumptive zygotes were collected from the oviducts approximately 16 h after hCG injection. Animal usage was approved by the Institutional Animal Care and Use Committee (05-0169). Cumulus enclosed zygotes were released into M2 medium (Sigma). Approximately 2/3 of embryos selected for cryopreservation were washed 3 times in M2. The remaining 1/3 were released into M2 medium containing 80 IU/ml hyaluronidase to remove the cumulus cells. The denuded embryos were washed 3 times with M2 medium and 3 times with K^+ modified simplex optimized medium (KSOM) (Specialty Media, Lavellette, NJ, USA) before they were transferred into a 10-μl drop of KSOM under mineral oil.

Vitrification protocol: Vitrification of cumulus-enclosed zygotes was conducted using a nylon mesh holder (pore size 60 μm; Sefar America Inc., USA) as described [24]. Cumulus-enclosed zygotes were used in order to reduce loss through the nylon mesh. A stepwise vitrification procedure was adopted using an ethylene glycol-Ficoll-sucrose (EFS) vitrification solution [13]. In this method, the cumulus enclosed zygotes were exposed for 7 min to 100 μl droplets of solution A, composed of 10% (v/v) ethylene glycol (EG), 4.5% (w/v) Ficoll-70 (F70) and 0.075 M sucrose (S) in modified Dulbecco's Phosphate Buffered Saline (PBS). Next, zygotes were exposed for 2 min to 100 μl droplets of solution B, consisting of 20% (v/v) EG, 9.0 % (w/v) F70 and 0.15 M S in PBS and finally they were immersed for 1 min in 100 μl droplets of solution C, composed of 40% (v/v) EG, 18% (w/v) F70 and 0.3 M S (EFS40) in PBS in 35 mm culture dishes. After equilibration, 30 to 40 cumulus enclosed zygotes at room

temperature were loaded onto the nylon mesh holder, which was placed on filter paper to remove excessive solution, immediately transferred to 2 ml cryovials and directly plunged into Liquid N_2 (LN_2) within 40 to 60 sec. Embryos were stored for 2 h in LN_2. After 2 h of storage in LN_2, the embryos were ultra rapidly thawed, by maintaining in air for 5 sec and then cryoprotectants were removed in a stepwise manner at 37°C. The nylon mesh was transferred from LN_2 into 2.7 ml of the warm PBS with a sequential series of 0.5, 0.25 and 0.125 M S dilutions by placing in each solution for 1, 2 and 3 min, respectively and finally transferring into PBS for 3 min in culture dishes. After warming, the nylon mesh was transferred into M2 medium containing 80 IU/ml hyaluronidase to remove the cumulus cells. The denuded zygotes were washed3 times with M2 medium and 3 times with KSOM medium before they were transferred into a 10-µl drop of KSOM under mineral oil.

Assessment of embryo survival: Viability of warmed embryos was determined based on visual examination of the integrity of embryo membrane, zona pellucida and the normality of the cytoplasm immediately after warming. During in vitro culture, embryo development was evaluated every 24 h, starting on Day 2 (48 h post-hCG). Two- to 8-cells, morula, early, expanded and hatched blastocysts stages were recorded.

Expression of cell death regulatory genes during preimplantation development: Pools of pronuclear stage embryos which are not exposed to vitrification protocol, embryos that succumbed to the vitrification process and 2-cell to blastocyst stage embryos from both control and vitrified groups were analyzed for quantitative expression of *Fas L*, *Fas*, *Bax*, *Bcl2* and *survivn* genes associated with apoptosis. The patterns of expression of the above genes were determined by reverse transcription polymerase chain reaction (RT-PCR).

Isolation of RNA: Embryos (n = 435) were collected, washed 3 times in PBS and then transferred to a 0.5 ml tube in as small a volume as possible. Thirty zygotes, 15 normally developed embryos of each stage (2-cell, 4-cell, 8-cell, morula and blastocyst) and 40 fragmented embryos after freeze thaw were put into separate tubes. The embryos were snap frozen in LN_2 and stored at ˉ80°C before analysis. Total RNA was isolated from embryos using Absolutely RNA Microprep Isolation Kit (Strategene, La Jolla, CA, USA) according to the manufacturer's instruction. Briefly, embryos were resuspended in 100 µl of the Lysis Buffer on ice and vortexed or pipetted repeatedly to facilitate the lysis of the embryos and release of RNA. An equal volume of 70% ethanol precipitation recovered total nucleic acid.

Table 1: Primers used for real time PCR

Genes	Gene Bank ID	Tm (°C)	Primer sequences (sense/antisense)
ß actin	NM_007393.1	59	5'CCAGTTCGCCATGGATGAC3'
			3'ATGCCGGAGCCGTTGTC 5'
Fas L	NM_010177.2	59	5'AAGAAGGACCACAACACA
			AATCTGT3'
			3'AACCAGAGCCACCAGAACCA5'
Fas	NM_007987.1	59	5'CCAGAAGGACCTTGGAAAATCA3'
			3'TCCAGACATTGTCCTTCATTTTCA5'
Bax	NM_007527	59	5'GGCCTTTTTGCTACAGGGTTT3'
			3'GTGTCTCCCCAGCCATCCT5'
Bcl2	NM_009741	59	5'AAGGGCTTCACACCCAAATCT3'
			3'TTCTACGTCTGCTTGGCTTTGA5'
Survivin	NM_009689	58	5'GGAGGCTGGCTTCATCCA3'
			3'AAAAAACACTGGGCCAAATCA5'

Reverse Transcription-Polymerase Chain Reaction: RNA was immediately converted to cDNA as described below. The reverse transcriptase reaction was performed with random primers using High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). A 20 µl reverse transcription reaction was set up by mixing 2 µl; of 10X RT Buffer, 0.8 µl of 25 X dNTPs, 2 µl of 10 X Random Primers, 1 µl of Multiscribe Reverse Transcriptase (50 U/µl) and 14.2 µl of RNA sample 14.2 µl at 25°C for 10 min, followed by 37°C for 120 min. Each reverse transcription reaction contained of 40 ng RNA.

Real-time PCR of the above cDNA was carried out in an ABI Prism 7700 sequence detector using Power SYBR Green (Applied Biosystems) as the detector, essentially following the manufacturer's instructions. All primer sequences (Table 1) were designed using the primer express 3.0 software (Applied Biosystems). The thermal cycling profile used was as follows: holding for 10 min at 95°C followed by 40 cycles of denaturation for 15 sec at 95°C and final extension for 60°C for 60 sec. Dissociation curve analysis was performed for each gene to ensure the specificity of PCR products. Gene expression was quantified by the 2-ddCt method [25]. Each analysis was performed on groups of 15 to 40 embryos and results are representative of 3 replicates on separate pools of embryos.

Statistical Analysis: The zygote survival subsequent to post vitrification-thawing, number of morphologically

damaged embryos, fragmented embryos during culture and the developmental rates of embryos at different stages based on the cleaved embryos subjected to culture was determined. Development score and percent of blastocyst that hatched were analyzed using mixed model (PROC MIXED in SAS 9.1). The proportion of cultured embryos that fragmented, the proportion that progressed to 2-cells, the proportion of 2-cell embryos that progressed to 4-cell, 8-cell, morula and blastocyst and the proportion of blastocysts that hatched for the two treatments were tested by Chi Square. The relative abundance of gene expression was subjected to one-way ANOVA using the general linear model (PROC-GLM in SAS 9.1). The significance was assigned at $P < 0.05$.

RESULTS

The morphological survival of vitrified pronuclear stage embryos is shown in Table 2. Recovery rate of zygotes after thawing was 93.1%. Immediately after thawing, 81.7 % of the zygotes survived the process of vitrification and warming based on morphological examination. A degeneration rate of 18.3 % was observed subsequent to the thawing process. Degenerated zygotes disintegrated into a poorly defined mass of debris; none of them cleaved during in vitro culture and the fragmentation rate was significantly higher ($P < 0.05$) than for controls. The embryos that survived the process of thawing and returned to culture showed significantly ($P < 0.05$) higher rate of fragmentation (10.0 %) than that in the control (4 %). The rate of development of zygotes to the blastocyst stage in the non-vitrified group was significantly ($P < 0.05$) higher than the corresponding rates in the vitrified group (Table 3).

Differential expression of apoptotic genes in vitrified embryos: One purpose of the experiment was to quantify the differences in the expression of gene transcripts involved in apoptosis at different stages of development of vitrified embryos. *Fas L* and *Fas* were inconsistently expressed among the tested samples, while Dax and survivin were consistently expressed (Fig. 1). In contrast, *Bcl2* mRNA could not be detected in any sample. *Fas L* mRNA was expressed in 4-cell, morula and blastocyst stages, whereas *Fas* mRNA was detected in both 4-cell and morula stage embryos. The expression of *Fas L* and *Fas* in the 4-cell embryos was 3.9 and 2.3 fold relative to control, respectively. Similarly a slight increase (1.1 fold) in the *Fas L* and *Fas* expression was detected in the morulae. A 2.2 fold increase in the *Fas L* alone was found in the blastocysts.

The relative abundance of *Bax* mRNA increased ($P<0.05$) in the vitrified embryos compared to the untreated control. There was a significant 3.86 ($P<0.05$) and 2.81 ($P<0.05$) fold increase in *Bax* mRNA levels in the 2-cell and 4-cell embryos, respectively. *Bax* mRNA levels did not change significantly ($P>0.05$) in the morula and blastocyst stages compared to untreated controls. The relative abundance of *survivin* did not differ significantly ($P>0.05$) among the different stages of embryonic development in the vitrified group compared with controls; transcript levels varied slightly among the different stages of embryos.

Expression of apoptotic genes in embryos undergoing fragmentation: Transcript analysis of fragmented embryos was carried out to test whether any change in apoptosis related genes occurred in embryos subjected to

Table 2: Mean (±SE) recovery, morphological survival rate and fragmentation of mouse zygotes after vitrification and thawing

	Embryos collected	Recovery from vitrification medium		Post thaw Recovery from mesh		Post thaw survival		Post thaw fragmentation		Fragmentation in culture	
Treatment	n	n	%	n	%	n	%	n	%	n	%
Nylon Mesh Vitrification	1469	1381	94.0±2.7	1286	93.1±4.4	1051	81.7±6.7	235	18.3±6.7	105	10.0±2.5[a]
Control	733	-	-	-	-	-	-	-	-	29	4.0±4.3[b]

[a-b] Values with different superscripts within column are significantly different at ($P < 0.05$)

Table 3: Post vitrification development (mean ± SE) of mouse zygotes using nylon mesh after 5 d of culture

	Embryos Cultured	1-cell		2-cell*		4-cell		8-cell		Morula		Blastocyst		Hatched blastocyst	
Treatment	n	n	%	n	%	n	%	n	%	n	%	n	%	n	%
Nylon Mesh	1051	135	12.8±0.001[a]	811	77.2±0.001[a]	642	79.2±0.002[a]	585	72.1±0.002[a]	548	67.6±0.002[a]	486	59.9±0.002[a]	186	38.3±0.002
Control	773	82	10.6±0.001[b]	662	85.6±0.001[b]	593	89.6±0.002[b]	561	84.7±0.002[b]	518	78.3±0.002[b]	438	66.2±0.002[b]	168	38.4±0.002

Values with different superscripts within each column are significantly different at ($P < 0.05$)

*Further cleavage from 4-cell stage to blastocyst stage is based on the number of 2-cells cleaved; hatched blastocyst rates are based on the total blastocysts

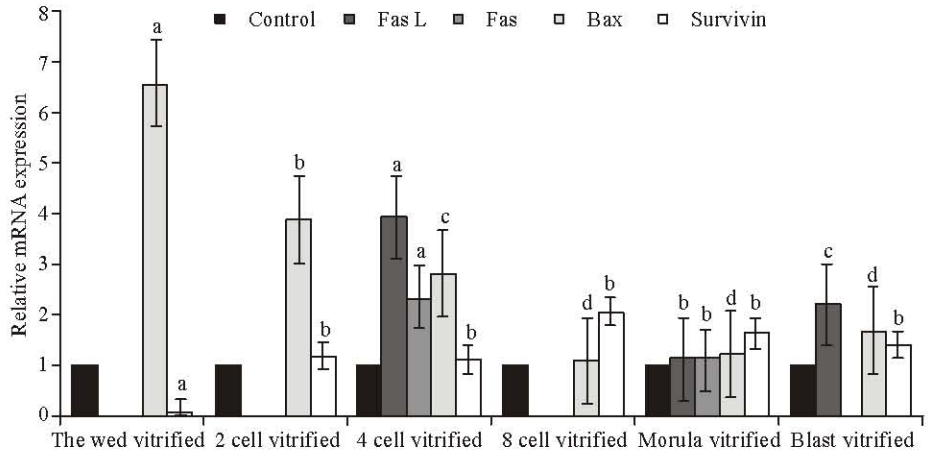

Fig. 1: Differential expression of apoptotic genes at various pre-implantation stages of development of vitrified mouse zygotes. Data are shown as mean ± SEM; for each gene means with different superscripts differ at $P < 0.05$

environmental changes consequent to vitrification and thawing. The gene expression of the embryos that had undergone fragmentation after vitrification and thawing was compared to the untreated zygotes. As expected, there was a robust increase in *Bax* mRNA levels in the embryos after vitrification and thawing; a typical 6.56 fold increase was found. Our results indicated that vitrification and thawing resulted in 95% decrease in the *survivin* mRNA level.

DISCUSSION

The results demonstrate that nylon mesh vitrification can result in high developmental rates for mouse zygotes. A simple and inexpensive technique for vitrification and post-thaw recovery of large numbers of cumulus intact pronuclear stage embryos was developed using nylon mesh. This is the first report of vitrification of cumulus intact mouse zygotes. The results for cleavage (77.2%) and blastocyst (59.9%) development of vitrified zygotes in this study were similar to those of other investigators [26, 27] using different systems. Conversely, the results obtained with vitrified germinal vesicle-stage cumulus oocyte complexes [28, 29] were much lower than the present report, possibly because the presence of the meiotic spindle in oocytes is usually thought to make them more susceptible to damage compared with that of zygotes [30].

Furthermore, potential hazards of contamination can be completely avoided by this method since there is no direct contact between the biological sample and LN_2 since the plastic wall of the cryovial represents an isolating layer between the LN_2 and the nylon mesh containing the embryos. Recently Kuwayama *et al.* [31] described a similar beneficial effect with Cryo Tip™ method for vitrification of human embryos.

Successful cryopreservation involves an adequate approach to minimize toxic, osmotic and other injuries during cryopreservation. Vitrification is a non-equilibrium cryopreservation method that needs relatively high concentration of cryoprotectants. Stepwise additions of cryoprotectants are beneficial for alleviating the toxic or major cell volume expansion effects exerted by cryoprotectants at high concentration [32]. Our high survival rates using murine zygotes and limited stepwise additions of cryoprotectants support the high survival rates of bovine blastocysts after vitrification using a 16-step method with permeable cryoprotectants [33].

The large mounting area of nylon mesh used simplified handling for mounting and recovery of embryos and was effective for the prevention of cumulus intact zygote damage as described [24]. The present study demonstrated the beneficial effect of addition of Ficoll and sucrose in the vitrification medium, in conjunction with stepwise vitrification procedure on subsequent development of zygotes to the blastocyst stage. Supplementation of saccharides such as Ficoll and sucrose in the vitrification medium could reduce the toxicity to the embryos by reducing the extra-cellular concentration of the cryoprotectant [34].

The present study demonstrated the co-expression of *Fas L*and *Fas* genes in the 4-cell and morula stages. The simultaneous expression of *Fas L* and *Fas* indicate that *Fas-Fas L* signaling pathway of apoptosis may be

operational in early embryos. A similar co-expression result for *Fas* and *Fas L* mRNA in 4-cell embryos was reported [35, 36] for mouse and human embryos, respectively. We demonstrated the expression of two major regulatory apoptotic genes, *Bax* and *survivin*, through all developmental stages. None of the samples tested showed the presence of *Bcl2* gene expression. A similar failure of detection of *Bcl2* gene expression in viable human embryos was reported by Liu *et al.* [37], suggesting the failure of detection of *Bcl2* may be due to the lack of sensitivity of the detection method or mRNA for *Bcl2* might have undergone degradation, thereby becoming virtually undetectable. We observed that transcription of the *Bax* gene was higher in the 2- and 4-cell embryos, while the levels were lower in the later stages of development. Yet, the relative abundance of *survivin* was not altered in 2- and 4-cell stages, but the values were higher in the later stages of development. It is possible that proapoptotic activity of *Bax* could be held in check by regulatory activity of increased levels of *survivin* after the 4-cell stage as suggested by Exley *et al.* [18] for *Bax* and *Bcl2*.

To our knowledge this is the first report of stage dependant developmental alterations in the expression of apoptotic genes following vitrification.

Based on the patterns observed in *Bax* and *survivin* in embryos that survived cryopreservation and thawing, we analyzed transcript accumulation in the embryos that had undergone fragmentation after thawing. It was interesting to note that expression of large amounts of *Bax* mRNA, but barely detectable amounts of *survivin* mRNA in these classes of embryos together with their total failure to progress in culture indicating that the balance of expression of these two genes may play a role in the survivability of pre-implantation embryos. In a similar study Jurisicova *et al.* [38] explained that the balance of expression of cell death promoter and protector genes might regulate cell death and embryo fragmentation might be attributable to a failure to achieve a balance of expression that favors embryo survival. The fate of the cell is determined by the ability of the cell death suppressors to sequester and hence neutralize the actions of the cell death inducers. Hence, the relative concentration of pro- and anti-apoptotic genes determines whether a cell lives or dies [39].

In conclusion, nylon mesh provides an efficient and attractive method of vitrification of large numbers of cumulus intact murine zygotes. We provide strong evidence that fragmented embryos consequent to vitrification degenerate through an apoptotic cell death mechanism after warming and there is increase in the relative abundance of apoptosis related genes in the vitrified pre-implantation embryos. The nylon mesh vitrification technique is promising and may be efficiently used for cryopreservation of large number of pronuclear stage embryos.

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