Transcript analysis of *Heat shock protein 72* and *protein 53* of 4-cell mouse embryos following Cryotop vitrification

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ABSTRACT: The effects of two different concentrations of cryoprotectants on survival and developmental capacity of four-cell mouse embryos were compared by Cryotop vitrification to demonstrate that lower concentrations provide the same results as higher previously reported concentrations with lesser negative molecular impact on embryo cells. For this latest, embryos were compared via transcript analyses of *Heat shock protein 72* (*Hsp72*) and *protein 53* (*p53*). Four-cell embryos were obtained from superovulated female mice and randomly assigned to one of three following groups: (*i*) control (non-vitrified), (*ii*) vit_1 (15% v/v: 7.5% ethylene glycol (EG) and 7.5% dimethyl sulfoxide (DMSO), and (*iii*) vit_2 (30% v/v: 15% EG + 15% DMSO). The cells vitrified by Cryotop were thawed and side-by-side to the control group divided into two parts: one part was used to analyze the morphological traits, survival rate, and embryo cleavage ability to form blastocysts, and the other part was examined for changes in transcript levels of *Hsp72* (*Hspa1a* + *Hspa1b*), *p53*, and *Hprt1* (reference gene) by quantitative Real-Time polymerase chain reaction (qPCR). The results were analyzed by One Way Analysis of Variance and the mean values compared with LSD ($P < 0.05$). The relative expression of *p53* in vit_2 (30% v/v) was significantly higher than in vit_1 (15% v/v) and in vit_1 it was higher than in the control. The relative expression of *Hsp72* was the same in vit_1 and vit_2 and significantly higher than in the control. The survival, cleavage, and blastocyst rates were the same for both vitrification treatments and significantly lower than in the control group. The up-regulations of *Hsp72* and *p53* following vitrification were suggestive of imposed heat shock, cold stress, and DNA damage to the mouse 4-cell embryos.

Keywords: cryopreservation; *in vitro* culture; murine; Real-Time PCR

Vitrification is a rapid cryopreservation method, enabling conversion from liquid to solid state, during which viscosity of the vitrifying solution is being extremely elevated due to the high concentration of cryoprotectant agents (CPAs) (Kasai, 1997; Massip, 2001).

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Since the first successful vitrification of mouse embryos in 1985 (Rall and Fahy, 1985), vitrification is routinely being used in the artificial reproductive technologies (ARTs). With no or little damage, vitrification has provided the chance to maintain scientifically important stocks, strains, and lines (exotic or endangered species). Moreover, import and export of embryos and gametes, and the storage of embryos for extended periods of time have been made possible via vitrification. The latter allows preservation of embryos for production of healthy offspring (Moore and Bonilla, 2006; Sheehan et al., 2006). Furthermore, cryopreservation of embryos will aid in the elimination of vertically transmitted diseases in animals and also would be beneficial to breeding programs. For example, the embryos of certain dogs such as guide dogs, drug-detecting dogs, and quarantine dogs can be kept and used in breeding programs (Abe et al., 2011). The necessity to use vitrification has led the researchers to seek a variety of approaches to improve the technique.

Changes in type and concentration of cryoprotectant, changes in the vitrification instrument shape, and application of stepwise cooling-warming are among many modifications that have been used so far (Vajta and Kuwayama, 2006). For instance, by comparing four cryoprotectants including DMSO, glycerol, polyethylene glycerol, and ethylene glycerol in 2–4 cell mouse embryos, it was revealed that ethylene glycol was the optimal cryoprotectant for the cryopreservation (Liu et al., 2011). Use of small volumes of cryoprotectant, i.e. less toxic material, via harbouring miniature tools increases the cooling and warming rates (Kasai, 2002; Hiraoka et al., 2004; Vajta and Kuwayama, 2006). Further to reducing the volume of cryoprotectants, lowering the concentration of CPAs may help to lessen their potential toxicity, improve post-thaw survival rate, and promise healthy developmental status (Tan, 2004; Katkov and Pogorelov, 2007; Yavin et al., 2009). Given that the optimization of the biophysical and biochemical conditions of the cells that undergo vitrification is important, the study of the vitrified cells on the molecular level may reveal the probable side-effects of such treatments.

Amongst a variety of exploratory molecular techniques, transcript analysis, gene-based or global analysis, provide some insights in the early development (Di Pietro et al., 2010; Habibi et al., 2010), better understanding of how to treat the cells to minimize the probable side-effects of vitrification and somewhat demonstrates the well-being of the cells during and subsequent to the cryopreservation (Mamo et al., 2006). For example, transcript analysis of histone deacetyltransferase 1 expression in mouse demonstrated a reduced expression upon cryopreservation indicating a probable negative impact on embryo development (Li et al., 2011). Here, to address the possible molecular alterations of the embryos at the four-cell stage, the transcript level of *Hsp72* (*Hspa1a + Hspa1b or Hsp70.3*) and *p53* (*Trp53*) were put under scrutiny as target genes via RT-PCR and the transcript data were calculated against *Hprt1* (Mamo et al., 2007, 2008).

Heat shock proteins (*HSPs*) are known to play an important role in various stress conditions, apoptosis, and zygotic gene activation (Burel et al., 1992). *HSPs* also appear to be involved in other developmental and/or differentiation steps of the cell in both normal and stress conditions. Previous reports were indicative of the presence of *Hsp72/Hsp73* in most embryonic stages (Kim et al., 2002; Liu et al., 2004).

Protein 53 (*p53*), a transcription factor, functions as a guardian of germ-line genomic integrity and the regulator of reproduction in vertebrates (Hu, 2009). In mouse embryos, the expression level of *p53* is high until the mid-gestation stage (Schmid et al., 1991) and the loss of *p53* in female mice causes reduced fertility (Choi and Donehower, 1999; Hu et al., 2007). Morover, *p53* is an important mediator of the responses to cellular stress and temperature change, transactivating and transrepressing a large number of target genes (Jin and Levine, 2001; Vousden and Lu, 2002).

Here, in a continuation of our previous studies (Habibi et al., 2010, 2011), a lower concentration of CPAs (7.5% EG and 7.5% DMSO) next to the higher concentration reported earlier by Kuwayama and Kato (2000) (15% EG and 15% DMSO) was applied to vitrify four-cell embryos and compared with non-vitrified cells at the same developmental stage both at the molecular level and morphological features. In comparison with blastocysts, four-cell mouse embryos have smaller volume and therefore one can accommodate more on vitrification tools such as Cryotop and further to this note it requires shorter period of time to collect them. Consequently and since no previous reports on transcript analysis of four cell embryos were available, it was decided to study the behaviour of these cells in response to vitrification. *HSP72* (*Hspa1a + Hspa1b)* and *p53*, stress-related candidate genes

that appear at early stages of embryo development, were chosen to be transcriptionally evaluated in mentioned conditions here in.

MATERIAL AND METHODS

All chemicals were purchased from Sigma Chemical Co. (St. Louis, USA) unless it has been stated.

CD1 (ICR) female mice aged 8–10 weeks and male mice aged 10–12 weeks (Lisbon University, Portugal) were housed in polycarbonate cages (12 h light/dark; 22 ± 2°C), and were fed with standard food and fresh water.

In all procedures, mice were handled according to the rules stipulated by the Animal Care in Portugal.

Preparation of 4-cell embryos

Female mice were superovulated by intraperitoneal injection of 10 IU pregnant mare serum gonadotropin (PMSG), followed by 10 IU of human chorionic gonadotropin (hCG) with a 48 h interval. Female and male mice (1 : 1) were mated and checked for vaginal plugs the next morning. The plug-positive female mice were killed humanely by cervical dislocation at 56–58 h post-hCG injection (Uechi et al., 1999; Sheehan et al., 2006), and 4-cell embryos were collected by flushing oviducts into KSOM (Millipore, Bedford, USA) supplement with 4 mg/ml BSA and 20mM Hepes buffer (Moore and Bonilla, 2006; Mukaida and Takahashi, 2007).

Study groups

The embryos from the mice sacrificed on each day were pooled and divided randomly into three groups: (*i*) control (non-vitrification), (*ii*) vit_1 (15% v/v: 7.5% EG and 7.5% DMSO), and (*iii*) vit_2 (30% v/v: 15% EG + 15% DMSO). Finally, 179 embryos were evaluated for survival, cleavage and blastocyst rates in vitrified and control groups. Embryos (270) were assessed for the expression of *Hsp72* (*Hspa1a* + *Hspa1b*), *p53*, and *Hprt1.*

For gene expression, each embryo pool containing 10 embryos was stored at –80°C in a minimum volume (2 ml) of RNase free water (Mamo et al., 2007).

Vitrification and thawing solutions

Modified Dulbecco's phosphate-buffered saline solution (PB_I) containing 10% (v/v) fetal bovine serum (FBS) (GIBCO, Grand Island, USA) was used as the basal medium or washing solution. The equilibration solution (ES) contained 7.5% (v/v) EG and 7.5% (v/v) DMSO in basal medium.

There were two vitrification solutions (VS) for two vitrified groups; VS_1: 7.5% (v/v) EG, 7.5% (v/v) DMSO, and 0.5 mol/l sucrose in basal medium and VS_2: 15% (v/v) EG, 15% (v/v) DMSO and 0.5 mol/l sucrose in basal medium. Thawing solution (TS) contained 0.5M sucrose and diluents' solutions (D_1, D_2, D_3, D_4, D_5) contained 0.4, 0.3, 0.2, 0.1, and 0.05M sucrose in basal medium, respectively.

Vitrification and thawing

Mouse 4-cell embryos were vitrified in two concentrations of vitrification solutions using Cryotop. Embryos were placed in three droplets of ES for 1 min total for all of the drops at 25°C. Subsequently, embryos were transferred in vitrification solution, VS_1 and VS_2, respectively for less than 30 s. Embryos were moved on the Cryotop (< 1 µl vitrification solution) and the Cryotop was immediately submerged in filter-sterilized liquid nitrogen and kept for at least 7 days. Samples were thawed by plunging the Cryotop into 1 ml of TS at 37°C for 1 min. Rehydration and gradual removal of cryoprotectants were performed in D1, D_2, D_3, D_4, and D_5 for 3 min at every step. Thawed embryos were washed three times in basal medium for 5 min at 25°C.

Definition of morphological survival

Embryos were defined "morphologically survived", if they possessed an intact *zona pellucida*, blastomeres, and refractive cytoplasm (Boonkusol et al., 2006; Mamo et al., 2006; Azadbakht and Rezazadeh Valojerdi, 2008). Following the thawing and cryoprotectant removal steps, four-cell embryos in 100 µl of sterilized $KSOM^{+AA}$ (Millipore, Bedford, USA) supplemented with 4 mg/ml BSA were incubated under mineral oil with the availability of 5% (v/v) CO_2, 5% (v/v) O_2, and 90% (v/v) N_2 for 1 h at 37°C.

The validity of morphological classification was confirmed by vital staining with 0.4% sterilized

trypan blue solution, a plasma membrane specific dye, in Hanks' balanced salt solution (Alexandra et al., 2007). The embryos were examined under an inverted micromanipulation microscope (Eppendorf, USA). The dead cells were stained dark blue by trypan blue, but viable cells were able to repel the dye and did not stain. The live cells were counted and recorded as survival rates. Visually dead embryos were discarded, and the morphologically intact embryos were cultured and the gene expression pattern was analyzed.

Embryo culture

The survived embryos in control, vit_1, and vit_2 groups were cultured in 20 µl droplets of $KSOM^{+AA}$ supplemented with 4 mg/ml BSA under mineral oil at 37°C in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2 to develop into the blastocyst stage. Embryos were assessed daily to record cleavage and blastocyst formation rates for 3 days.

Gene expression and Reverse transcription reaction (RT)

The relative quantification of gene transcripts was carried out by Real Time-PCR. Super Script™ III Platinum® Cells Direct Two-Step qRT-PCR Kit with SYBR® Green (Invitrogen, Carlsbad, USA) was used to carry out cDNA synthesis and PCR.

Embryos were lysed in 1 µl lysis enhancer and 10 µl resuspension buffer for every PCR tube, which were incubated at 75°C for 10 min in a Thermal Cycler 9700 (Applied Biosystems, Foster City, USA). To degrade any contaminating DNA, the cell lysates were treated with 5 µl DNase I and 1.6 µl DNase I buffer (10×) at 25°C for 5 min. The embryos were treated with 4 µl of 25mM EDTA and incubated at 70°C for 10 min. For first-strand cDNA synthesis, 20 µl 2× RT Reaction Mix and 2 µl RT Enzyme Mix were added to each tube and incubated at 25, 50, and 85°C for 10, 20, and 5 min, respectively.

Real-Time Polymerase Chain Reaction (qPCR)

The primer pairs for each gene were designed, synthesized, and validated by Molecular Diagnostic Companies (MDC, Burgess Hill, UK). The primer sequences, annealing temperatures, and GenBank accession numbers are provided (Table 1). Since designing gene specific primers for either *Hspa1a* or *Hspa1b*, orthologs of human *Hsp72*, was almost impossible, the designed primer set considers the total changes of both transcripts at the same time.

Real-Time PCR was conducted in a Real-Time Cycler 7500 (Applied Biosystems, Foster City, USA). To confirm the specificity and integrity of the PCR products, melting curve analyses were performed for all Real-Time PCR reactions. Standard curves were generated using serial dilutions of cDNAs.

Real-Time PCR was conducted for cDNAs and standards in triplicates with two no template controls (NTC). Reactions (25 µl) contained 12.5 µl Platinum® SYBR® green qPCR super mix-UDG (uracil-DNA glycosylase), 0.5 ml Rox Reference dye, 0.5 µl primer mix (sense and antisense primers, 300nM each), 6.5 µl autoclaved distilled water, and 5 µl of cDNA in each well.

Cycling parameters were 50°C for 2 min (UDG incubation), 95°C for 2 min, followed by 50 cycles of 95°C for 15 s and 60°C for 30 s. The melting curve was analyzed at 95°C for 15 s and temperature lowered to 60°C for 15 s. Each experiment was repeated three times.

The data were analyzed with the integrated ABI 7500-V2.0.1 software (Applied Biosystems, Foster City, USA) and were normalized with *Hprt1* within the log linear phase of the amplification curve using the comparative Cycle-Threshold (Ct) method. The relative expression ratios (R) of *Hsp72* (*Hspa1a* + *Hspa1b*) and *p53* were estimated based on a ΔCt formula (Pfaffl, 2001; Luciano et al., 2007; Avci et al., 2008). PCR efficiencies (Pfaffl, 2001; Luciano et al., 2007) of the genes ranged between 1.98–2.0. ΔCt was the difference between the Ct values of controls and samples.

Statistical analysis

One Way Analysis of Variance was performed on the average percentages of survived, cleaved embryos, blastocyst formation, and relative amount of *Hsp72* (*Hspa1a* + *Hspa1b*) and *p53* mRNAs in control, vit_1, and vit_2 groups. Following analysis of variance, mean values were compared. The level of significance was set at less than 0.05 and LSD (Least Significant Difference) test was used to compare the means.

Table 1. Primers and conditions used for quantification of gene expression by Real-Time PCR

Gene symbol	GenBank accession No.	Sense primer (5'-3')	Anti-sense primer (5'-3')	Melting temperature (°C)	Amplicon size (bp)
Hsp72	NM_010479	5'ACGGCATCTTCGAGGTGAA 3'	5' TGTTCTGGCTGATGTC-CTTCT 3'	50	129
p53	NM_011640	5'GAACCGCCGACCTATC-CTTA3'	5'GCACAAACACGAACCT-CAAA3'	52	90
Hprt1	NM_013556	5'TCCTCCTCAGAC-CGCTTTT3'	5'AGGTATA-CAAAACAAATCTAGGTCAT3'	48	118

RESULTS

First set of experiments: survival, cleavage, and blastocyst formation

To find out the effects of different concentration of cryoprotectants on developmental rates, embryos were vitrified in two vitrified groups (vit_1 and vit_2) and the survival, cleavage (to morula stages), and blastocyst formation rates were assessed and compared with each other and with the control group.

No significant difference was found in survival rate between vit_1 and vit_2 while the survival rate of control group was significantly higher than in vitrified groups ($P < 0.05$) (Table 2).

The cleavage rates were 70.2% ± 1.5, 65% ± 1.1, and 61% ± 1.7 in control, vit_1, and vit_2 groups, respectively (Table 3). The blastocyst formation rates were 45.6% ± 1.1, 42.5% ± 1, and 36.6% ± 1.2 in control, vit_1, and vit_2 groups, respectively (Table 3).

The cleavage and blastocyst development rates of vitrified embryos were significantly lower than non-vitrified embryos ($P < 0.05$) while there were no differences in vit_1 and vit_2 groups.

Second set of experiments: gene expression

The effects of different concentrations of cryoprotectants on the expression of *p53* and *Hsp72* (*Hspa1a* + *Hspa1b*) in 4-cell embryos were ana-

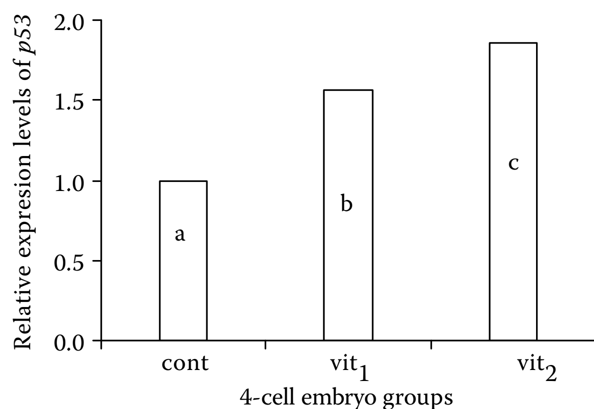

Figure 1. Relative quantification of *p53 (Trp53)* after normalization by *Hprt1* in three groups: cont = control (non-vitrified) group, vit_1 = vitrification with 7.5% DMSO and 7.5% EG, vit_2 = vitrification with 15% DMSO and 15% EG. Mean comparison was performed by Least Significant Difference (LSD) at $P < 0.05$. a, b, and c indicate the significant differences between control, vit_1, and vit_2

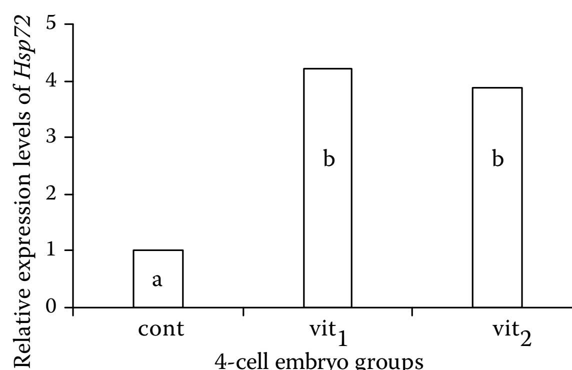

Figure 2. Relative quantification of *Hsp72* (*Hspa1a* + *Hspa1b*) after normalization by *Hprt1* in three groups: cont = control (non-vitrified) group, vit_1 = vitrification with 7.5% DMSO and 7.5% EG, vit_2 = vitrification with 15% DMSO and 15% EG. Mean comparison was performed by Least Significant Difference (LSD) at $P < 0.05$. a and b indicate the significant differences between control with vit_1 and vit_2

Table 2. Survival rates of 4-cell embryos (n = 179) in control and vitrified groups were recorded after 1-h incubation at 37°C in sterilized KSOM^{+AA} supplemented with 4 mg/ml BSA

Group	Concentration of cryoprotectans	Total No. of embryos	No. of survived embryos	Mean of survival rate (%)	Standard deviation
Cont	0%	64	57	89.1^a	0.11
Vit$_1$	15% (7.5% EG + 7.5% DMSO)	56	40	71.4^b	0.11
Vit$_2$	30% (15% EG + 15% DMSO)	59	41	69.5^b	0.12

cont = control (non-vitrified) group, vit$_1$ = vitrification with 7.5% DMSO and 7.5% EG, vit$_2$ = vitrification with 15% DMSO and 15% EG, DMSO = dimethyl sulfoxide, EG = ethylene glycol

^{a,b}significant difference between control and vitrified groups, mean comparison was performed by Least Significant Difference test at P < 0.05

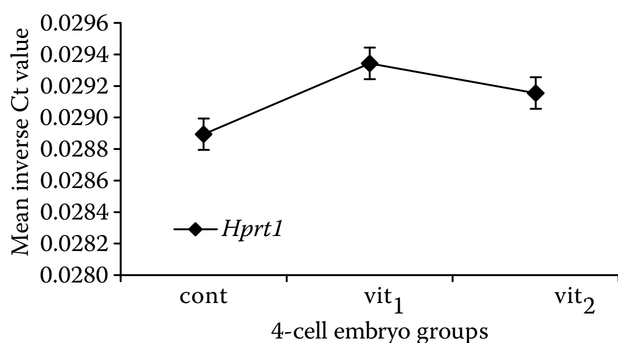

Figure 3. Mean inverse Ct values of *Hprt1* as the relevant abundance of transcript in three groups: Ct = threshold cycle, cont = control (non-vitrified) group, vit$_1$ = vitrification with 7.5% DMSO and 7.5% EG, vit$_2$ = vitrification with 15% DMSO and 15% EG. Bars are indicative of having no significant difference, i.e. *Hprt1* is a suitable gene for analysis of vitrification

lyzed with RT-PCR and the data were normalized against *Hprt1*.

In comparison to control, significant up-regulations for *p53* (1.6 and 1.9 fold) and *Hsp72* (4.2 and 3.9 fold) were seen for vit$_1$ and vit$_2$, respectively (P < 0.05) (Figure 1 and 2). The transcript levels of *Hsp72* (*Hspa1a* + *Hspa1b*) for vitrified 4-cell embryos, vit$_1$ and vit$_2$, had no significant difference with each other (P > 0.05) (Figure 2). However, normalized relative expression ratio of *p53* demonstrated significant difference between vit$_1$ and vit$_2$ (P < 0.05) (Figure 1).

Mean inverse Ct values of *Hprt1* had no significant differences in vitrified and control groups (P > 0.05) (Figure 3).

DISCUSSION

Transcript analysis of *Hsp72* (*Hspa1a* + *Hspa1b*) and *p53* showed up-regulations in vitrified groups once compared to the control, similar to the previous results following other vitrification methods (Boonkusol et al., 2006), which were indicative that the cells have experienced a stress situation.

Abiotic stresses such as thermal stress can affect a suite of genes that may alter the cellular physiology and even sometimes lead to apoptosis.

Table 3. Cleavage and blastocyst rates of 4-cell embryos (n = 179) in control and vitrified groups were recorded on a daily basis and incubated in sterilized KSOM^{+AA} supplemented with 4 mg/ml BSA at 37°C

Group	Concentration of cryoprotectans	No. of survived embryos	No. of cleavaged embryos (%)	No. of blastocysts (%)
Cont	0%	57	40 (70.2)^a	26 (45.6)^a
Vit$_1$	15% (7.5% EG + 7.5% DMSO)	40	26 (65)^b	17 (42.5)^b
Vit$_2$	30% (15% EG + 15% DMSO)	41	25 (61)^b	15 (36.6)^b

cont = control (non-vitrified) group, vit$_1$ = vitrification with 7.5% DMSO and 7.5% EG, vit$_2$ = vitrification with 15% DMSO and 15% EG, DMSO = dimethyl sulfoxide, EG = ethylene glycol

^{a,b}significant difference between control and vitrified groups, mean comparison was performed by Least Significant Difference test at P < 0.05

Among which, *HSP*s have been demonstrated to be good candidates to underpin that the cell(s) have experienced the stress and to what degree the outside force was effective on the cell(s). In regards to heat stress, particular cellular functions such as DNA synthesis, transcription, downstream processing of RNA molecules and translation can be influenced. Moreover, some drastic changes may occur to the cytoskeleton and membrane permeability as a result of thermal stress (Kuhl and Rensing, 2000). Meanwhile, cells undergoing vitrification treatment not only experience thermal stress but an osmotic stress may impact them. Similarly, this latter stress may affect the DNA integrity (Huang et al., 2008).

The relative expression of *p53* in vit_1 was significantly lower than in vit_2 but no significant difference was seen for *Hsp72* (*Hspa1a* + *Hspa1b*) between vit_1 and vit_2 in 4-cell embryos. In another experiment, it was noted that in 2-cell mouse embryos the expression pattern for *Hsp72* (*Hspa1a* + *Hspa1b*) remained stable in vit_1 as opposed to 4-cell embryo following vitrification. Furthermore, the survival and developmental rates of 2-cell embryos in vit_1 were better than in vit_2 (data not shown). Therefore, it can be stated that the developmental stage of embryos is very important to tolerate the cryoprotectant's concentration during vitrification. Meanwhile, it can be concluded that the advancement in developmental stage may have improved some physiological systems that withstand the stress better than earlier stages such as 2-cell embryo. This notion can be explained through the expression pattern of *Hsp72* (*Hspa1a* + *Hspa1b*) that remained stable between both vitrification treatments at 4-cell stage, i.e. cells experienced less stress with concentration change in cryoprotectants. On the other hand, the application of cryoprotectants affects some other genes such as *p53* that may be used as more sensitive kinds of indicators of the stress conditions imposed to the cells with rather different molecular pathway. Here and based on the transcript analysis of *p53*, it can be concluded that the 4-cell mouse embryos have experienced thermal stress during vitrification and thawing steps.

A compromise in morphological traits, the survival, cleavage, and blastocyst rates, was noted for the 4-cell embryos that had undergone vitrification, once compared with the control group. Our finding was similar to the earlier results reported by other groups (Fugger et al., 1988; Selick et al., 1995; Uechi et al., 1997, 1999; Li et al., 2011). Sensitivity of young embryos to chilling and heightened osmolarity (Uechi et al., 1999; Kasai, 2002; Baltz and Tartia, 2010), toxicity of cryoprotectants, and the change of stress-related genes (Figures 1 and 2) are some of the causes that may lead to this compromise. In a vitrification study by Zhang et al. (2009) based on Kuwayama et al. (2007), the blastocyst formation rate of the 2-cell embryos was significantly lower than of vitrified 4- and 8-cell embryos, suggesting the higher sensitivity of younger embryos. Further, some embryos, e.g. hamster embryos, are quite sensitive to be taken out of their natural niche causing a drastic effect on survival rate (Kasai, 2002).

Considering the morphological traits, vit_1 and vit_2 performed similarly, while examining the transcript level of *p53* indicates otherwise. This may suggest that the superficial studies do not always tell the whole story and sheer cut between live and dead cells would not provide enough information concerning molecular changes occurring within the cells. In brief, the need for detailed analyses of genes, proteins, and even metabolic pathways might be a necessity in ARTs and more specifically in vitrification with the use of cryoprotectants. Meanwhile, since no differences were noted between vit_1 and vit_2 in morphological traits and *p53* was affected less in vit_1, it can be concluded that lowering the cryoprotectant concentration may lead to better results. This may be the consequence of lesser damage to the cell membrane and some other organelles during the dehydration step in the presence of lower concentrations of solutes surrounding the cell (Aman and Parks, 1994).

Finally, earlier reports have been indicative of successful use of the Cryotop vitrification for mammalian embryos in the presence of 15% EG and 15% DMSO (Kuwayama and Kato, 2000; Kuwayama et al., 2005a, b; Kuwayama, 2007; Cobo et al., 2008; Almasi-Turk et al., 2009). This technique is greatly influenced by the developmental stages of embryos, type of species and/or even the animal strain (Tsutsumi et al., 1998; Ueichi et al., 1999; Moore and Bonilla, 2006; Morato et al., 2008). As a result a thorough modification of the technique is required concerning the above factors to obtain it for the better results with minimal consequences.

CONCLUSION

The objective of this work was to find out an improved method by studying the *in vitro* viability,

development, and selected gene expression of 4-cell stage mouse embryos following vitrification in a comparison of two different cryoprotectant concentrations (15% vs. 30%) in the vitrification solution. Combinations of dimethyl sulfoxide (DMSO) and ethylene glycol (EG) were used as cryoprotectants and the Cryotop was implemented as the carrier to investigate the effects of vitrification on selected gene expression of mouse 4-cell embryos by the Real-Time PCR.

Our results demonstrated that Cryotop vitrification worked relatively well with minimal effect but significant to the control on survival and developmental rates. The expression of *HSP72* (*Hspa1a* + *Hspa1b*) remained the same for both treatments, but significantly higher than the control indicating that regardless of cryoprotectant concentration embryos experienced stress condition. Nevertheless, the expression of *p53* was lower in vit_1 as opposed to vit_2 that was suggestive of slightly less stressed condition provided by lowering the cryoprotectant concentration.

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REFERENCES

Abe Y., Suwa Y., Asano T., Ueta Y.Y., Kobayashi N., Ohshima N., Shirasuna S., Abdel-Ghani M.A., Oi M., Kobayashi M., Miyoshi K., Suzuki H. (2011): Cryopreservation of canine embryos. Biology of Reproduction, 84, 363–368.

Alexandra I., Telea A.D.A., Caraba V., Pacala N. (2007): Experimental tries to establish the preimplantational mammalian embryos viability throughout staining. Zootehnie şi Biotechnologii, 40, 111–116.

Almasi-Turk S., Roozbehi A., Aliabadi E., Haeri A., Sadeghi Y., Hosseini A. (2009): Developmental consequences of mouse cryotop-vitrified oocyte and embryo using low concentrated cryoprotectants. Iranian Journal of Reproductive Medicine, 7, 181–188.

Aman R.R., Parks J.E. (1994): Effects of cooling and rewarming on the meiotic spindle and chromosomes of *in vitro*-matured bovine oocytes. Biology of Reproduction, 50, 103–110.

Avci M.E., Konu O., Yagci T. (2008): Quantification of Slit-robo transcripts in hepatocellular carcinoma reveals two groups of genes with coordinate expression. BMC Cancer, 8, 392–402.

Azadbakht M., Rezazadeh Valojerdi M. (2008): Development of vitrified-warmed mouse embryos co-cultured with polarized or non-polarized uterine epithelial cells using sequential culture media. Journal of Assisted Reproduction and Genetics, 25, 251–261.

Baltz J.M., Tartia A.P. (2010): Cell volume regulation in oocytes and early embryos: connecting physiology to successful culture media. Human Reproduction Update, 16, 166–176.

Boonkusol D., Gal A.B., Bodo S., Gorhony B., Kitiyanant Y., Dinnyes A. (2006): Gene expression profiles and *in vitro* development following vitrification of pronuclear and 8-cell stage mouse embryos. Molecular Reproduction and Development, 73, 700–708.

Burel C., Mezger V., Pinto M., Rallu M., Trigon S., Morange M. (1992): Mammalian heat shock protein families. Expression and functions. Cellular and Molecular Life Sciences, 48, 629–634.

Choi J., Donehower L.A. (1999): *p53* in embryonic development: maintaining a fine balance. Cellular and Molecular Life Sciences, 55, 38–47.

Cobo A., Kuwayama M., Perez S., Ruiz A., Pellicer A., Remohi J. (2008): Comparison of concomitant outcome achieved with fresh and cryopreserved donor oocytes vitrified by the Cryotop method. Fertility and Sterility, 89, 1657–1664.

Di Pietro C., Vento M., Guglielmino M.R., Borzi P., Santonocito M., Ragusa M., Barbagallo D., Duro L.R., Majorana A., De Palma A., Garofalo M.R., Minutolo E., Scollo P., Purrello M. (2010): Molecular profiling of human oocytes after vitrification strongly suggests that they are biologically comparable with freshly isolated gametes. Fertility and Sterility, 94, 2804–2807.

Fugger E.F., Bustillo M., Katz L.P., Dorfmann A.D., Bender S.D., Schulman J.D. (1988): Embryonic development and pregnancy from fresh and cryopreserved sibling pronucleate human zygotes. Fertility and Sterility, 50, 273–278.

Habibi A., Farrokhi N., Moreira-da-Silva F., Bettencourt B.F., Bruges-Armas J., Amidi F., Hosseini A. (2010): The effects of vitrification on gene expression in mature mouse oocytes by nested quantitative PCR. Journal of Assisted Reproduction and Genetics, 27, 599–604.

Habibi A., Hosseini A., Farrokhi N., Amidi F., Carvalhais I., Chaveiro A., Moreira-da-Silva F. (2011): Successful vitrification of mouse oocytes using the cryotop method with moderate cryoprotectant concentrations. Canadian Journal of Animal Science, 91, 385–388.

Hiraoka K., Hiraoka K., Kinutani M., Kinutani K. (2004): Blastocoele collapse by micropipetting prior to vitrification gives excellent survival and pregnancy outcomes

for human day 5 and 6 expanded blastocysts. Human Reproduction, 19, 2884–2888.

Hu E., Feng Z., Teresky A.K., Levine A.J. (2007): *p53* regulates maternal reproduction through LIE. Nature, 450, 721–724.

Hu W. (2009): The role of *p53* gene family in reproduction. Cold Spring Harbor Perspectives in Biology, doi: 10.1101/cshperspect.a001073.

Huang J.Y., Chen H.Y., Park J.Y., Tan S.L., Chian R.C. (2008): Comparison of spindle and chromosome configuration in *in vitro-* and *in vivo-*matured mouse oocytes after vitrification. Fertility and Sterility, 90,(Suppl. 4), 1424–1432.

Jin S., Levine A.J. (2001): The *p53* functional circuit. Journal of Cell Science, 114, 4139–4140.

Kasai M. (1997): Cryopreservation of mammalian embryos. Molecular Biotechnology, 7, 173–179.

Kasai M. (2002): Advances in the cryopreservation of mammalian oocytes and embryos: development of ultrarapid vitrification. (Review). Reproductive Medicine and Biology, 1, 1–9.

Katkov I.I., Pogorelov A.G. (2007): Influence of exposure to vitrification solutions on 2-cell mouse embryos: II. Osmotic effects or chemical toxicity? CryoLetters, 28, 409–427.

Kim M., Geum D., Khang I., Park Y.M., Kang B.M., Lee K.A., Kim K. (2002): Expression pattern of *HSP25* in mouse preimplantation embryo: heat shock responses during oocyte maturation. Molecular Reproduction and Development, 61, 3–13.

Kuhl N.M, Rensing L. (2000): Heat shock effects on cell cycle progression. Cellular and Molecular Life Sciences, 57, 450–463.

Kuwayama M. (2007): Highly efficient vitrification for cryopreservation of human oocytes and embryos: the Cryotop method. Theriogenology, 67, 73–80.

Kuwayama M., Kato O. (2000): All round vitrification of human oocytes and embryos. Journal of Assisted Reproduction and Genetics, 17, 477.

Kuwayama M., Vajta G., Ieda S., Kato O. (2005a): Comparison of open and closed methods for vitrification of human embryos and the elimination of potential contamination. Reproductive BioMedicine Online, 11, 608–614.

Kuwayama M., Vajta G., Kato O., Leibo S.P. (2005b): Highly efficient vitrification method for cryopreservation of human oocytes. Reproductive BioMedicine Online, 11, 300–308.

Li J.J., Pei Y., Zhou G.B., Suo L., Wang Y.P., Wu G.Q., Fu X.W., Hou Y.P., Zhu S.E. (2011): Histone deacetyltransferase1 expression in mouse oocyte and their *in vitro*-fertilized embryo: effect of oocyte vitrification. CryoLetters, 32, 13–20.

Liu C.H., Yang C.C., Lin D.P., Wu M.H., Tsai K.J. (2004): Stored of Hsp72/Hsp73 in germinal vesicle-stage mouse oocytes. Reproduction in Domestic Animals, 39, 19–24.

Liu W.X., Lu H., Luo M.J., Xu L.Z. (2011): Effects of different cryoprotectants and cryopreservation protocols on the development of 2–4 cell mouse embryos. CryoLetters, 32, 240–247.

Luciano P., Bertea C.M., Temporale G., Maffei M.E. (2007): DNA internal standard for the quantitative determination of hallucinogenic plants in plant mixtures. Genetics, 1, 262–266.

Mamo S., Bodo S., Kobolak J., Polgar Z., Tolgyesi G., Dinnyes A. (2006): Gene expression profiles of vitrified *in vivo* derived 8-cell stage mouse embryos detected by high density oligonucleotide microarrays. Molecular Reproduction and Development, 73, 1380–1392.

Mamo S., Gal A.B., Bodo S., Dinnyes A. (2007): Quantitative evaluation and selection of reference genes in mouse oocytes and embryos cultured *in vivo* and *in vitro*. BMC Developmental Biology, 7, 14.

Mamo S., Gal A.B., Polgar Z., Dinnyes A. (2008): Expression profiles of the pluripotency marker gene *POU5F1* and validation of reference genes in rabbit oocytes and preimplantation stage embryos. BMC Developmental Biology, 9, 67.

Massip A. (2001): Cryopreservation of embryos of farm animals. Reproduction in Domestic Animals, 36, 49–55.

Moore K., Bonilla A.Q. (2006): Cryopreservation of mammalian embryos: the state of the art. Annual Review of Biomedical Sciences, 8, 19–32.

Morato R., Izquierdo D., Paramio M.T., Mogas T. (2008): Cryotops versus open-pulled straws (OPS) as carriers for the cryopreservation of bovine oocytes: effects on spindle and chromosome configuration and embryo development. Cryobiology, 57, 137–141.

Mukaida T., Takahashi K. (2007): Vitrification of day 2–3 human embryos: using various techniques (Cryoloop, Cryotop, and conventional cryostraw). In: Tucker M.J., Liebermann J. (eds): Vitrification in Assisted Reproduction. Informa Healthcare, London, UK, 183–193.

Pfaffl M.W. (2001): A new mathematical model for relative quantification in real time RT-PCR. Nucleic Acids Research, 29, 2002–2007.

Rall W.F., Fahy G.M. (1985): Ice-free cryopreservation of mouse embryos at –196°C by vitrification. Nature, 313, 573–575.

Schmid P., Lorenz A., Hameister H., Montenarh M. (1991): Expression of *p53* during mouse embryogenesis. Development, 113, 857–865.

Selick C.E., Hofmann G.E., Albano C., Horowitz G.M., Copperman A.B., Garrisi G.J., Navot D. (1995): Embryo quality and pregnancy potential of fresh compared with

frozen embryos – is freezing detrimental to high quality embryos? Human Reproduction, 10, 392–395.
Sheehan C.B., Lane M., Gardner D.K. (2006): The CryoLoop facilitates re-vitrification of embryos at four successive stages of development without impairing embryo growth. Human Reproduction, 21, 2978–2984.
Tan J. (2004): Vitrification of human oocytes and bovine oocytes and embryos. PhD Diss. Westmount, Quebec: Selwyn House School.
Tsutsumi O., Uechi H., Sone H., Yonemoto J., Takai Y., Momoeda M., Tohyama C., Morita M., Taketani Y. (1998): Presence of dioxins in human folicular fluid: their stage-specific action on the development of preimplantation mouse embryos. Biochemical and Biophysical Research Communications, 18, 498–501.
Uechi H., Tsutsumi O., Morita Y., Taketani Y. (1997): Cryopreservation of mouse embryos affects later embryonic development possibly through reduced expression of the glucose transporter *GLUT1*. Molecular Reproduction and Development, 48, 496–500.
Uechi H., Tsutsumi O., Morita Y., Takai Y., Taketani Y. (1999): Comparison of the effects of controlled-rate cryopreservation and vitrification on 2-cell mouse embryos and their subsequent development. Human Reproduction, 14, 2827–2832.
Vajta G., Kuwayama M. (2006): Improving cryopreservation systems. Theriogenology, 65, 236–244.
Vousden K.H., Lu X. (2002): Live or let die: the cell's response to *p53*. Nature Reviews Cancer, 2, 595–604.
Yavin S., Aroyo A., Roth Z., Arav A. (2009): Embryo cryopreservation in the presence of low concentration of vitrification solution with sealed pulled straws in liquid nitrogen slush. Human Reproduction, 24, 797–804.
Zhang J., Cui J., Ling X., Li X., Peng Y., Guo X., Heng B.C., Tong G.Q. (2009): Vitrification of mouse embryos at 2-cell, 4-cell and 8-cell stages by cryotop method. Journal of Assisted Reproduction and Genetics, 26, 621–628.

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