Cytoskeletal alterations in different developmental stages of *in vivo* cryopreserved preimplantation murine embryos

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Background: This study aimed to investigate the effects of vitrification and slow freezing on actin, tubulin, and nuclei of *in vivo* preimplantation murine embryos at various developmental stages using a Confocal Laser Scanning Microscope (CLSM).

Material/Methods: Fifty female mice, aged 4–6 weeks, were used in this study. Animals were superovulated, cohabitated overnight, and sacrificed. Fallopian tubes were excised and flushed. Embryos at the 2-cell stage were collected and cultured to obtain 4- and 8-cell stages before being cryopreserved using vitrification and slow freezing. Fixed embryos were stained with fluorescence-labelled antibodies against actin and tubulin, as well as DAPI for staining the nucleus. Labelled embryos were scanned using CLSM and images were analyzed with Q-Win software V3.

Results: The fluorescence intensity of both vitrified and slow-frozen embryos was significantly lower for tubulin, actin, and nucleus as compared to non-cryopreserved embryos (p<0.001). Intensities of tubulin, actin, and nucleus in each stage were also decreased in vitrified and slow-frozen groups as compared to non-cryopreserved embryos.

Conclusions: Cryopreservation of mouse embryos by slow freezing had a more detrimental effect on the actin, tubulin, and nucleus structure of the embryos compared to vitrification. Vitrification is therefore superior to slow freezing in terms of embryonic cryotolerance.

Key words: cytoskeletal damage • cryoinjury • cryopreservation • vitrification • slow freezing • preimplantation embryos

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Background

A normal cytoskeleton, composed of microfilaments, intermediate filaments, and microtubules, is critical for cell functions and embryo development [1]. As dramatic as cell death, damage to the cytoskeleton renders the affected cell non-viable and the cell will not completely develop and survive [2,3]. This is because the cytoskeleton gives strong support to the cell plasma membrane and maintains intracellular organelle organization [4,5]. Due to its interaction with the cell membrane, the cytoskeleton may also have a significant role in determining post-dehydration and post-thaw viability.

The cytoskeleton is linked almost continuously to the cell membrane [6]. It is capable of modifying membrane structure [7], altering shape or morphology of the membrane [8,9], changing the mechanical properties of the cell [10], and influencing the membrane water transport [11,12]. Cytoskeletal alterations in cryopreserved embryos are attributed to many confounding factors such as developmental stage during cryopreservation, the origin of embryos (*in vivo* or *in vitro*), the type of cryoprotectant, selection of cryopreservation methods, and degree of zona absence [13]. Therefore, any alterations and disruptions in cytoskeleton may explain most of the detrimental effects in cell morphology and organelle distributions of any freezing-induced methods [14].

The storage of gametes could provide a powerful tool in research and contributes to advances in mammalian infertility treatment, the conservation of endangered species, and reproductive biology, as well as increasing the availability of oocytes for research applications such as genetic engineering or embryo cloning [15]. Therefore, cryopreservation of oocytes and embryos is an integral part of assisted reproductive technology (ART). During the past few decades, various methods of oocyte and embryo cryopreservation have been standardized. Among these, vitrification is considered a viable method to preserve oocytes and embryos [16]. The success of embryo cryopreservation is normally gauged by morphological observation and developmental capacity [17,18].

It is important to study the ultrastructural damage of the embryos during cryopreservation because the ultrastructural components of mouse embryos undergo dramatic architectural changes, considerable stress [19], and morphological and functional damage during cryopreservation. In many cases, the damage can reduce embryo viability due to destabilization and disruption of cell organelles [20,21] and the cytoskeleton [22,23]. Cryopreservation may also cause injuries to the cell, such as mitochondria and endoplasmic reticulum alterations, poorly developed desmosomes, and lack of tight junctions [24,25]. Therefore, by understanding the type of cytoskeletal damage from vitrification and slow freezing, new strategies might be developed and implemented to improve cryopreservation techniques.

Embryos can be damaged during cryopreservation and thawing, either by the formation of large intracellular ice crystals or by increased intracellular concentration of solutes and accompanying changes that result from dehydration of cells during cryopreservation [19]. Damage may occur to embryos when they pass through a range of temperatures during cryopreservation. Early injuries may start at between +15°C to −5°C when the meiotic spindles, microtubules, and cytoplasmic lipid will be destroyed by the chilling effects. When the temperatures goes down to −5°C to −80°C, the formation of extracellular and intracellular ice crystals are the major cause of embryo damage. When the temperature goes to −150°C, fracture of the zona pellucida cannot be avoided [26].

Actin fibers provide a fundamental cytoskeletal framework in all cells [27]. Actin treadmilling provides a signal modulating nuclear gene expression and provides an additional regulatory twist to cell motility. Actin has been recently proposed to be an important factor throughout the entire RNA biogenesis pathway as a component of chromatin remodeling complexes [28]. During morphogenesis, actin plays a major role in cellular movements, including ingression, epiboly, invagination, involution, and delamination. All of these cell movements involve remodeling of the actin cytoskeleton [29]. Therefore, any changes and disruption in actin structures will jeopardize the survival of embryos, perhaps because the cryopreservation process may cause actin depolymerization that completely blocks chromosome migration [30,31].

Recent improvements of florescence dyes and microscopy techniques allow detailed examination of specific cellular structures as microfilaments, microtubules, mitochondria, zona membranes, and nuclei. A confocal laser scanning microscope can be used to improve the selection of the most viable embryos by detecting the degree of cytoskeletal and ultrastructural damage of the embryos following cryopreservation. Therefore, the aim of this study was to document cellular damage after cryopreservation of embryos using slow freezing and vitrification and to evaluate the cellular sensitivity to cryopreservation. Information on cytoskeletal damage may provide a good indication of cryotolerance and enhances the efficiency of embryonic cryopreservation.

Material and Methods

Experimental animals

Fifty female ICR mice aged 4–6 weeks and weighing 25–30 grams were used in this study. All animals were kept in standard laboratory conditions at 27°C with 12-h light-dark periods and they were given food pellets and water *ad libitum*. Ethics approval from the university Animal Care and Use Committee

(Code: ACUC-7/11) was obtained and all procedures used followed strictly the Malaysian animal ethics guidelines.

Origin, culture and harvest of embryos

Female mice were superovulated using pregnant mare's serum gonadotropin (PMSG) (5 IU/kg b.w.) and human chorionic gonadotropin (hCG) (5 IU/kg b.w.) hormones [Intervet, Holland] intraperitoneally 48 h later before being mated with the fertile male mice with a ratio of 1:1. Female mice with the presence of vaginal plugs were considered to be pregnant and were selected as embryo donors. The mice were then sacrificed by cervical dislocation 48 h post-copulation. Ovaries from each animal were placed into the petri dishes containing M2 medium [Sigma, USA], fallopian tubes were excised, and embryos were flushed under a dissecting microscope [Leica Zoom 2000, Japan]. Only good quality 2-cell stage embryos with an intact zona pellucida and symmetrical blastomeres were selected for the experiments. The selected 2-cell embryos were rinsed with M2 medium and cultured *in vitro* in 24-well plates [Orange Scientific, Belgium] filled with 100 µl of M16 media [Sigma, USA] overlaid with mineral oil [Sigma, USA] to further develop to 4- and 8-cell stages. Embryo culture was equilibrated in a water-jacketed 37°C CO_2 incubator (5% CO_2; 95% air) [Memmert, Germany] and the development of embryos was observed daily under the inverted microscope [Olympus 1X81 SF-3, Japan].

Vitrification and warming

The method of vitrification in this experiment was adopted from Nagy et al. (2003). Embryos were cryopreserved at the 2-, 4-, and 8-cell stages of development. In this experiment, a polymer called EFS40 was used as a cryoprotectant [18,32–34]. A boat made from a Styrofoam box with 1-cm thickness and grooves were used to place the straw during cooling procedures. At least 5 cm height of liquid nitrogen was filled in the Styrofoam box 30 min before the start of the experiment. Embryos were collected using M2 media and kept at room temperature. A 1-ml syringe fitted with a white pipette tip was used to load the EFS40 solution into the straw. Thirty microliters of EFS40, corresponding to 15 ml of solution, was pulled in, followed by a 5-mm air space. Then an additional 5 mm of solution was pulled in just after the air bubble near the opening. The straw was laid down carefully and a total of 10 embryos were aspirated in a minimal volume of M2 using a transfer pipette. The straw was then placed in a horizontal position at eye level. The transfer pipette was inserted into the horizontally held straw and the embryos were expelled into the EFS40, while smoothly withdrawing the pipette. The timer was set at 1 min when the embryos were in place. After that, the straw was sealed at the open end by using polyvinyl alcohol (PVA) powder and was wiped clean. Once the embryos were equilibrated with the cryoprotectant for 1 min at room temperature, the straw was placed on the groove in the Styrofoam boat floating on liquid nitrogen vapor. After that, it was immersed in liquid nitrogen and stored in a liquid nitrogen tank until use. During the warming procedure, the straw was removed from the liquid nitrogen and then placed on the Styrofoam boat. After being held in air, it was immersed in a 20°C water bath for 60 s. The straws were then removed from the water bath and wiped dry. The seal was cut and the contents expelled into a dish containing 0.5M sucrose. The embryos were then transferred into M2 media, rinsed twice in fresh M2 media, and placed in a M16 droplet overlaid with mineral oil for culture.

Slow freezing and thawing

The methods of slow freezing by Nagy et al. (2003) were used with some modifications. The embryos were also cryopreserved at 2-, 4-, and 8-cell stages of development. In this experiment, liquid nitrogen and a boat made from a 1-cm – thick Styrofoam box were used during the cooling and thawing procedures. Embryos were collected using M2 media and kept at room temperature. Embryos were then aspirated into the straw containing D-PBS and DMSO before being transferred into an ice bath for 30 min. The straw was then transferred to a –20°C freezer for 30 min before being placed in a –80°C freezer for 1 h. After that, the straw was placed on a Styrofoam boat in liquid nitrogen vapor. After 30 min, the straw was immersed and stored in liquid nitrogen until use. To thaw the embryos following slow freezing procedures, the straws containing embryos were removed from the liquid nitrogen tank and then placed on a Styrofoam boat for 15 min. When completely thawed, the seal was cut and the contents expelled into a dish containing D-PBS for washing. The embryos were then transferred into M2 media, rinsed twice with fresh M2 media and finally placed in M16 droplets covered with mineral oil for culture.

Immunofluorescent staining

A total of 300 mouse embryos at different stages of development were used. The cytoskeletal structures were stained by immunofluorescence staining. The embryos were fixed with 4% paraformaldehyde for 24 h and the nuclei were stained with 4',6-diamino-2-phenylindole dihydrochloride (DAPI) for 40 min before being permeabilized with 1% Triton-X in Phosphate Buffer Saline (PBS). After 10 min incubation in 1% Triton-X, actin structures were labelled with Alexa Fluor 635 and tubulin structures were labelled with Anti-α-tubulin for 1 h, the embryos were washed with PBS twice for 10 min and counterstained with DAPI for 40 min. The embryos were mounted on slides, sealed, stored in the dark at 4°C overnight and finally viewed under a Confocal Laser Scanning Microscope (CLSM) [Leica TCS SP5 AOBS, Germany]. Images taken from CLSM were converted to JPEG format before being analyzed using QWin Software V.3.
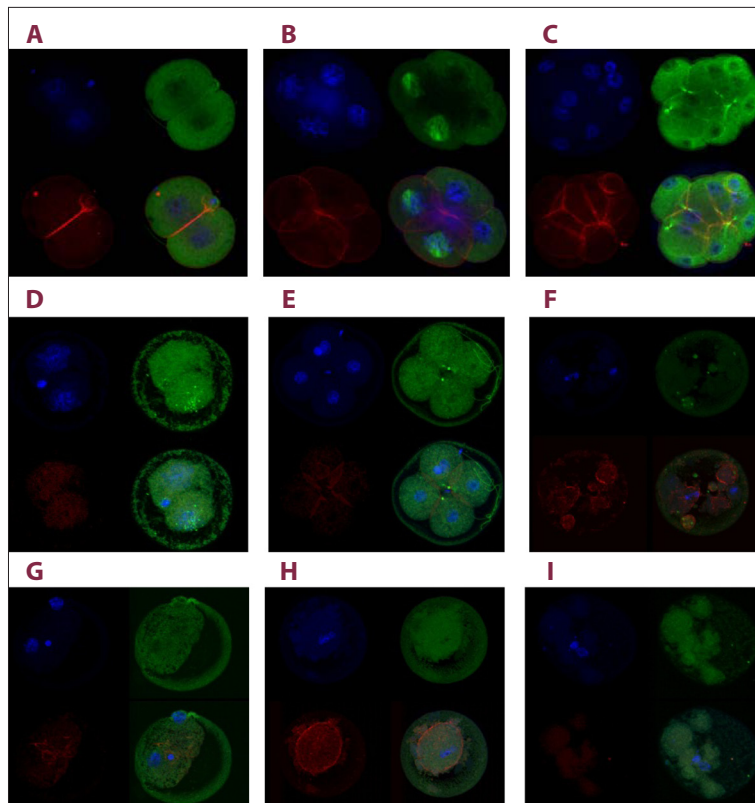
Figure 1. Cytoskeletal organizations of 2-, 4-, and 8-cell *in vivo* murine embryos. Fixed control (**A–C**), vitrified (**D–F**), and slow-frozen (**G–I**) embryos stained with Alexa Fluor 635 phalloidin; actin (red), Anti-α-tubulin (green), and DAPI; nucleus (blue) were observed under a confocal laser scanning microscope.

Statistical analysis

These experiments consisted of 50 samples for each stages of development. Assessment of cytoskeletal quality and cellular damage are based on the intensity of fluorescent probes after immunofluorescence staining and the morphology of the embryos. The percentage of embryo development until the hatched blastocyst indicates the survivability of the embryos. Intensities of fluorescent probes in each stage of embryos were compared between experimental groups using one-way ANOVAs with a Bonferroni correction. In all cases, statistics were performed using Statistical Package for Social Sciences version 16 (SPSS Inc, USA), and differences were considered significant when p<0.05.

Results

Confocal imaging studies of cryopreserved embryos

Effect of vitrification and slow freezing on actin, tubulin, and nucleus

The changes in actin and tubulin integrity in preimplantation embryos with and without prior cryopreservation were observed by using CLSM. Three different developmental stages – 2-, 4-, and 8-cell stage embryos – were chosen for the comparison between vitrification, slow freezing, and non-cryopreserved

embryos. Confocal images of embryos were analyzed by using QWin Software V3 and the images are shown in Figure 1A–1I.

Figure 1A–1C shows the controls (non-cryopreserved) embryos in 2-, 4-, and 8-cell stages. The vitrified embryos are shown in Figure 1D–1F and Figure 1G–1I shows the slow-frozen embryos in various developmental stages. Red color represents the actin structures of embryos stained with Alexa Fluor 635 phalloidin, green stain of anti-α-tubulin represents the tubulin structures, and the nucleus of embryos appeared blue in color when it stained with DAPI.

Fluorescence micrographs from Figure 1A–1I show the distribution of tubulin, actin, and nucleus clustering in controls, vitrified and slow-frozen, of 2-, 4-, and 8-cell stage embryos. The nuclei of individual cells were located in the center region of the cytoplasm of controls 2- and 4-cell (Figure 1A, 1B), as well as in vitrified 2- and 4-cell embryos (Figure 1D, 1E). Outward migration of the nucleus of the blastomere is observed in the control 8-cell embryos (Figure 1C). However, major disruption of the nucleus occurred in vitrified 8-cell embryos (Figure 1F) and all slow-frozen embryos (Figure 1G–1I).

Complete perinuclear clustering of tubulin with a thin green stain were observed in control embryos (Figure 1A–1C). In 8-cell embryos, the cortex area became strongly stained with anti-α-tubulin and perinuclear clustering was still observed. In the

Table 1. Fluorescence intensity of tubulin, actin, and nucleus in 2-, 4-, and 8-cell stages of mouse embryos (Mean ±SEM). Values with different superscripts are significantly different as compared to controls (p<0.001).

Developmental stage	Cytoskeletal structure	No. of embryos	Percentage of fluorescent intensity		
			Non-vitrified (control)	Vitrification	Slow freezing
2-cell	Actin	50	28.1±1.6[bc]	6.3±0.3[a]	6.2±0.2[a]
	Tubulin	50	49.5±1.5[bc]	21.8±1.0[ac]	11.4±0.5[ab]
	Nucleus	50	50.0±1.7[bc]	6.6±0.4[a]	3.6±0.3[a]
4-cell	Actin	50	32.5±1.0[bc]	16.0±1.0[ac]	4.2±0.3[ab]
	Tubulin	50	69.4±2.4[bc]	34.8±1.0[ac]	11.2±0.6[ab]
	Nucleus	50	52.0±1.4[bc]	14.0±0.7[ac]	3.8±0.3[ab]
8-cell	Actin	50	37.2±0.9[bc]	13.7±0.5[ac]	9.1±0.3[ab]
	Tubulin	50	76.5±2.0[bc]	42.9±1.2[ac]	19.9±0.9[ab]
	Nucleus	50	49.1±2.1[bc]	14.6±0.6[a]	9.3±0.3[a]

a – p<0.001, compared to control; b – p<0.001, compared to vitrification; c – p<0.001 compared to slow freezing.

vitrified groups (Figure 1D, 1E), the embryos display partial migration of tubulin into the subcortical region because of the deleterious effect of vitrification. However, more damage in tubulin was observed in the 8-cell stage (Figure 1F). Sizes of the perivitelline space were also increased in vitrified 2-and 4-cell embryos (Figure 1D, 1E) as well as in slow-frozen 2-cell embryos (Figure 1G).

The actin distributions were observed clearly in controls embryos (Figure 1A–1C). However, the fluorescent intensity seemed to decline slightly in all cryopreserved embryos (Figure 1D–1I) and the most damage were observed in vitrified and slow-frozen 8-cell stage embryos (Figure 1F and 1I).

Intensity of tubulin, actin, and nucleus of cryopreserved embryos

Percentage of probes intensities in each confocal image of control and cryopreserved embryos were calculated using QWin Software and the results are summarized in Table 1 and Figures 2–4.

Table 1 shows percentage of fluorescent intensities (%) of tubulin, actin, and nucleus in 2-, 4-, and 8-cell stages of the pre-implantation embryos following different cryopreservation (vitrification or slow freezing). In all cryopreserved groups, as the number of the cells increased, fluorescent intensities of tubulin, actin, and nucleus were decreased slightly as compared to non-vitrified (control) groups. The results clearly show that both cryopreservation methods caused highly significant changes in cytoskeletal structures of embryos, especially in tubulin, actin, and nucleus as compared to non-vitrified embryos (p<0.001). However, intensity comparisons showed that actin, tubulin, and nucleus intensities of vitrified embryos were slightly higher than in slow-frozen embryos.
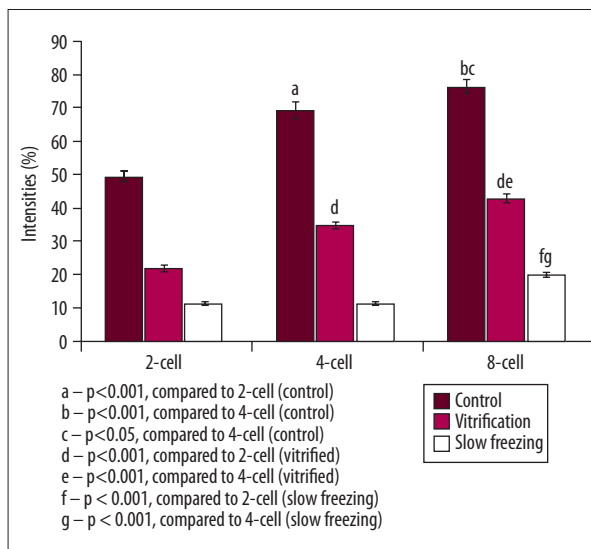

a – p<0.001, compared to 2-cell (control)
b – p<0.001, compared to 4-cell (control)
c – p<0.05, compared to 4-cell (control)
d – p<0.001, compared to 2-cell (vitrified)
e – p<0.001, compared to 4-cell (vitrified)
f – p < 0.001, compared to 2-cell (slow freezing)
g – p < 0.001, compared to 4-cell (slow freezing)

Figure 2. Comparison of tubulin intensity (%) between all treatment groups. Values with different superscripts are significantly different.

Percentage of tubulin, actin, and nucleus were also compared between each developmental stage and freezing techniques. Results show that vitrification methods caused highly significant changes in tubulin structures between 2-, 4-, and 8-cell stages (p<0.001). The percentages of intensities were also having significant difference between stages of development. However, the results were not significant between 4-cell and 8-cell stages in terms of nucleus intensities.

Figure 2 shows the percentage of tubulin intensities in control, vitrified, and slow-frozen groups. In the control (non-vitrified) group, the distributions of tubulin were increased as the number of the cells increased. The percentage of tubulin intensities in 2-, 4-, and 8-cell stage embryos were 49.5±1.5%,
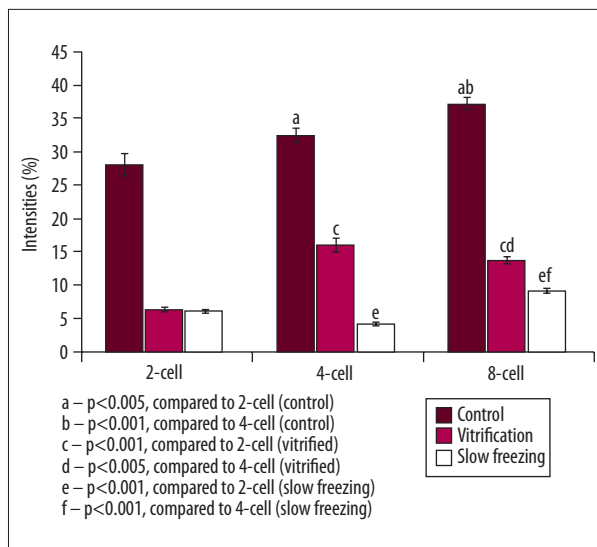
Figure 3. Comparison of actin intensity (%) between all treatment groups. Values with different superscripts are significantly different.

a – p<0.005, compared to 2-cell (control)
b – p<0.001, compared to 4-cell (control)
c – p<0.001, compared to 2-cell (vitrified)
d – p<0.005, compared to 4-cell (vitrified)
e – p<0.001, compared to 2-cell (slow freezing)
f – p<0.001, compared to 4-cell (slow freezing)

Figure 4. Comparison of nucleus intensity (%) within all treatment groups. Values with different superscripts are significantly different.

a – p<0.001, compared to 2-cell (vitrified)
b – p<0.001, compared to 2-cell (slow freezing)
c – p<0.001, compared to 4-cell (slow freezing)

the normal and vitrified embryos – 3.6±0.3%, 3.8±0.3%, and 9.3±0.3%, respectively.

69.4±2.4%, and 76.5±2.0%, respectively. This pattern also was seen in the vitrified and slow-frozen embryos but the increment was less than in the control embryos. In the vitrified group, the percentage of tubulin intensities were 21.8±1.0%, 34.8±1.0%, and 42.9±1.2%, respectively. The lowest intensities of tubulin were in the slow freezing group – 11.3±0.5%, 11.2±0.6%, and 19.9±0.9%, respectively.

Figure 3 shows the percentage of actin intensities in control, vitrified, and slow-freezing groups. As shown in the graph, the percentage of actin intensities in 2-, 4-, and 8-cell stages were 28.1±1.6%, 32.5±1.1%, and 37.2±0.9%, respectively. As seen in tubulin, the pattern of the actin intensity increased from 2- to 8-cell stage but there was more damage in the 4-cell stage in all treatment groups. However, during the 8-cell stage, the actin intensity continued to increase. In the vitrified group, the actin intensities in 2-, 4-, and 8-cell stages were 6.3±0.3%, 16.0±1.0%, and 13.7±0.5%, respectively. For the slow-freezing group, the intensity of actin was 6.2±0.2% in 2-cell stage and it decreased to 4.2±0.3% in the 4-cell stage, and then increased again in the 8-cell stage to 9.1±0.3%.

Figure 4 shows the percentage of nucleus intensities in control, vitrified, and slow-frozen groups. From the results of this study it can be concluded that the most damage is seen in the nucleus after vitrification and slow freezing. As the number of the cells increased, nuclear intensity increased, as observed with the intensities of tubulin and actin. Intensities of nuclei in the control group were 50.0±1.7%, 52.0±1.4%, and 49.1±2.1%, respectively. The intensities were decreased in the vitrified group – 6.6±0.4%, 14.0±0.7%, and 14.6±0.6%. However, the lowest intensities were in the slow-frozen embryos as compared to
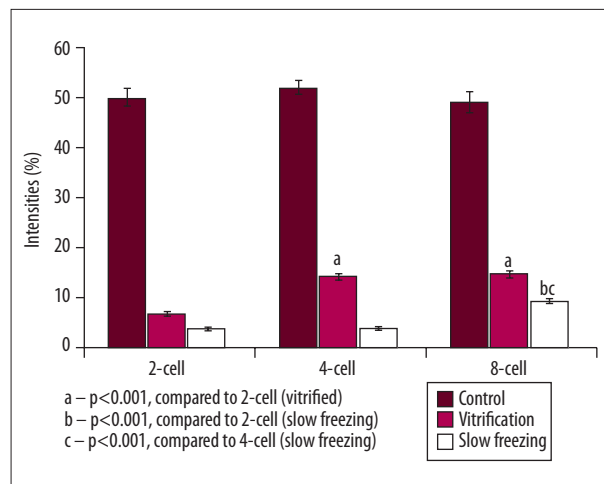
Discussion

In this study, the cytoskeletal organizations of the preimplantation murine embryos following different types of cryopreservation were observed. The results from confocal images clearly showed that cryopreservation by using vitrification and slow freezing methods caused significant percentage changes in fluorescent intensity of tubulin, actin, and nucleus integrity as compared to non-vitrified embryos (p<0.001). However, intensity comparisons showed vitrified embryos have higher fluorescent intensity in cytoskeletal organization as compared to slow-frozen embryos. The prominent changes in cytoskeletal organizations seen in slow freezing and vitrification may be due to the changes in metabolic function, developmental capacity, physiological necessities, and requirements of the embryos [35,36].

The cytoskeleton of a mammalian embryo is made of a unique and fragile intracellular network of microfilaments and microtubules that play major roles in normal function and development of the cells [20]. The cytoskeleton also plays a significant role in determining the cell's response to freeze/thaw and dehydration stresses, and cell survival. An intact cytoskeleton is also essential for cytokinesis and karyokinesis; therefore, if the cytoskeleton is irreversibly damaged, the mitotic cell cycle will end, rendering the affected cell non-viable. Moreover, mitotic arrest would result in a non-viable embryo [37].

Results from this study demonstrate that cryopreservation using vitrification and slow freezing methods caused deleterious damage in the embryo cytoskeleton, such as actin, tubulin, and

nucleus at various developmental stages of preimplantation embryos as compared to non-cryopreserved embryos. This result is in agreement with the earlier report by Skidmore et al. (2008), who found that the quality and distribution of the cytoskeleton was affected by the freezing technique employed and the embryo age/size. They also found that the effect was significantly lower in vitrified and conventionally frozen/thawed embryos, as compared to controls [37].

Microfilaments and microtubules are very sensitive to the cooling process [38–40], and their integrity is essential for proper progression through meiosis [41,42]. In fact, tubulin is responsible for the constitution of the meiotic spindle and drives the proper alignment of chromosomes on the metaphase plate, and microfilaments are necessary for cortical granule migration and polar body expulsion. The degree of repolymerization was critically dependent on the method used to dilute or remove the CPA, and failure to completely rehydrate resulted in an embryo with pyknotic nuclei and both a diminished magnitude and altered cellular distribution of microfilaments and microtubules [43].

In the present study, embryos at various developmental stages were cryopreserved with EFS40 at room temperature. EFS40 were used as a CPAs. EFS40 consists of both permeating and non-permeating cryoprotectant and it is used to cause a shift in the isotonic state between the intracellular and extracellular spaces, driving water out of the cell. Permeating cryoprotectant produces large-volume changes during freezing and thawing processes. Therefore, less damage occurred in vitrified embryos compared to slow-frozen embryos as the results of cryoprotective effect of EFS40.

Cells are protected by permeating agents such as EG and DMSO, which are organic solutes, during cooling and warming procedures prior to and after storage in liquid nitrogen [44]. Our findings shows that vitrification by using EFS40 as CPAs enhanced and gave better protection to 2- and 4-cell embryos in terms of cytoskeletal damage and embryo quality as compared to slow freezing. These findings confirmed that low toxicity of EFS40 is suitable to vitrify embryos, as reported by Chen et al. (2008). This finding also agrees with Nowshari and Brem (2001), who reported that EG-based solutions were the best cryoprotectant for embryo cryopreservation. Kuleshova et al. (2001) also found that inclusion of polymers such as Ficoll and sucrose improved embryo survivability after freezing. This may be because the amount of toxic cryoprotectants may be decreased by replacing some cryoprotectants with polymers and sugars. Ficoll has been used successfully with EFS40 solutions during cryopreservation and researchers found that it is suitable for cryopreservation of 8-cell and morula stage mouse embryos [45–47] and hatched blastocysts [48]. Vitrification leads to an extreme increase in viscosity in the presence of high concentration of

CPAs, permitting the transition of aqueous solutions into a glassy state, bypassing the crystalline state [49]. The concentration of CPAs required for vitrification has been calculated as being 4–7 times higher than that required in slow freezing, and may result in either osmotic or chemical toxicity [50,51].

Embryo cryopreservation depends critically on embryo size and developmental stage [52,53]. This stage-dependant sensitivity of embryos to damage during cryopreservation, and differences between species and freezing techniques, are major obstacles to the widespread application of cryopreservation in commercial practice [37]. Our study focused on the effect of vitrification and slow freezing in early preimplantation stages (2-, 4-, and 8-cell) of mouse embryos, as other researchers have demonstrated that early stage embryos can be vitrified [54–56]. However, Cseh et al. (1999) claimed that the most suitable developmental stage for vitrification was the compacted morulae. In this case, the present study is limited because cytoskeletal images of embryos at morulae and blastocyst stages cannot be analyzed by CLSM due to overlapping of the fluorescent signals. This limitation was also found in a previous study in which researchers had difficulty identifying the distribution of the cytoskeleton within each blastomere because the fluorescence signals overlapped [69].

Our results show that cryopreservation using slow freezing methods have more deleterious effects on the cytoskeletal structures. Our finding is in agreement with that of others who demonstrated that conventional slow freezing methods lead to moderate cytoskeleton disruption and cell death [43,58]. Dobrinsky et al. (2000) reported that vitrifying pig embryos caused considerable microfilament disruption and significantly reduced developmental competence. However, our results disagree with that of Luciano et al. (2009) who showed that the slow freezing method used on feline embryos preserves the cytoskeleton organization better than vitrification.

Other factors such as physical injuries by intracellular and extracellular ice formation, cryoprotectant toxicity, osmotic stress, and chilling injuries have been proposed to contribute to embryo damage after cryopreservation [37,59]. The formation of ice crystals that occurs in slow freezing methods disrupts the structure of the embryos during cryopreservation. During cryopreservation, ice crystals can develop within the cytoplasm of a cell and damage the cellular architecture or lyse the plasma membrane [37]. Studies performed on animal models as well as in humans have shown that exposure to low temperatures [60–63] and to CPAs [29,63] results in depolymerization of the microtubules, sometimes with attendant dispersal of chromosomes [64]. Previous studies have shown that changes in culture conditions can alter the organization or structure of mitochondria in the oocytes or embryos of mice [65]. Freezing can also denature or damage critical cellular organelles and

enzymes, thereby compromising the ability of the embryo to repair structural damage and continue development [14,21].

The actin structure also plays an important role in cells. Actin gives mechanical strength to the cell and plays a major role during cytokinesis, anchoring the centrosomes during mitosis and generating the cell shape. According to Skidmore et al. (2008), slow freezing and thawing of camel embryos resulted in a modest level of cell death but more widespread disruption of the actin cytoskeleton. Their findings agree with our results, which showed that slow freezing cause more disruption of actin structures of embryos as compared to controls and vitrified embryos.

The nucleus coordinates all cellular activities, contains the genetic material, and is crucial in the production of proteins. The present results show that the reduction of nucleus intensities is seen in vitrification and slow freezing. As the number of the cells increased, nuclear intensity increased, as observed with the intensities of tubulin and actin. Slow-frozen embryos showed the lowest intensity as compared to the normal and vitrified embryos. In this case, most of the chromatin structure may be disrupted in the embryos cryopreserved by the slow freezing method. Any disruption that occurs in chromatin distributions will then affect the formation of actin and tubulin and may be blocked the chromosome migration [30,31].

In addition, the extracellular space became hypertonic due to the increase in solutes compared with that of the intracellular spaces, resulting in water leaving the cell and subsequent cell shrinkage. During warming, a rapid influx of water into the cell occurred due to the increased concentration of solutes within the cell, before the permeating cryoprotectant can be removed. This resulted in osmotic shock and cell lysis. The entry of water is faster than the exit of solutes, thus the cell volume is increased beyond its lytic volume. Contraction of the cell membranes during cryopreservation caused a rearrangement of the cell organelles, including the cytoskeleton and the meiotic spindles, which may be a contributing factor to the decrease of tubulin intensities in the vitrified and slow-frozen embryos.

Conclusions

Based on confocal studies, the slow freezing method showed worse cryopreservation effects compared to vitrification in terms of cytoskeletal damage, which strongly suggests that vitrification is the better procedure for cryopreservation. Our study also found that cryopreservation of 2-cell-stage embryos causes the least cytoskeletal injury as compared with 4- and 8-cell stages.

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