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Early-Cleaving Embryos are Better Candidates for Vitrification: Patterns Associated with Mitochondria and Cytoskeleton

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ABSTRACT

This study was conducted to investigate mitochondrial, nuclear chromatin and cytoskeletal organisation of vitrified embryos based on timing of the first zygotic cleavage. Embryos were retrieved from superovulated ICR mice, 28 hours after hCG injection. Two-cell stage embryos were categorised as early-cleaving (EC), while zygotes with 2-pronuclei as late-cleaving (LC) embryos. Embryos were cultured overnight in M16 medium supplemented with 3% bovine serum albumin (BSA) in carbon dioxide incubator. After 20 hours, the embryos were vitrified for one hour and warmed to room temperature. They were then fixed and immunostained to visualise distribution and intensity of mitochondria, nuclear chromatin and cytoskeleton. Finally, the embryos were mounted on glass slides and examined under a Confocal Laser Scanning Microscope (CLSM). Fluorescence intensities were analysed using LAS-AF-Lite Software. Results showed that EC embryos had significantly higher mitochondria (39.22 ± 12.50 versus 35.42 ± 14.61 pixel, $p<0.05$) and actin filaments fluorescence intensities (11.43 ± 5.44 versus 5.23 ± 2.20 pixel) compared to LC embryos ($p<0.001$). There was no significant difference in nuclear chromatin and microtubules fluorescence intensities between EC and LC

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embryos. These findings suggest that greater cryosurvivability of vitrified EC compared to LC embryos was contributed by higher densities of mitochondria and actin filaments. Thus, selection of embryos for IVF procedure should be made based on timing of the first zygotic cleavage.

Keywords: Actins, chromatin, cytoskeleton, early cleavage, embryo, microtubules, mitochondria, vitrification

INTRODUCTION

Timing of the first zygotic cleavage has become a common selection parameter in many in vitro fertilization (IVF) laboratories (Fancsovits et al., 2005; Fu et al., 2009; Nielsen & Ali, 2010). Embryos that cleaved early were selected for embryo transfer because of their good quality and higher developmental viability (Lundin, Bergh, & Hardarson, 2001; Salumets et al., 2003; Van Montfoort, Dumoulin, Kester, & Evers, 2004; Fu et al., 2009; Isom, Li, Whitworth, & Prather, 2012). However, the factors contributing to the superiority of early cleavers are still not clear. It is hypothesised that superiority may be related to the mitochondria, nucleus and cytoskeletal structures of embryos. Previous study conducted by the researchers reported higher abundance of actin filaments and nuclear chromatin in early cleaving embryos compared to their late counterparts (Wan Hafizah et al., 2015). Nonetheless, the effects of EC and LC status on vitrification outcomes, due to differences in actin, tubulin, nuclear chromatin and mitochondria have not been elucidated.

Vitrification of embryos has become a widely used method in embryology laboratories (Konc, Kanyo, Kriston, Somoskoi, & Cseh, 2014). However, the uses of high concentration of cryoprotectant and very low temperature during vitrification negatively impacts intracellular organelles and structures including mitochondria, cytoskeleton and nuclear chromatin (Jain & Paulson, 2006). In a study conducted to investigate embryo survivability and viability after vitrification based on timing of the first zygotic cleavage, EC embryos were found to produce higher survivability and developmental viability compared to EC embryos, after vitrification (Jusof, et al., 2015). It is not clear whether organisation of cytoskeleton and other organelles such as mitochondria and nucleus in vitrified EC embryos contribute to their higher survivability as reported in non-vitrified EC embryos (Wan Hafizah, et al., 2015). In a review paper, Lechniak, Pers-Kamcyc and Pawlak (2008) hypothesised that timing of the first zygotic cleavage was related to the number of mitochondria within the oocytes.

Mitochondria are the most abundant organelles at the early stages of embryonic development. They play an important role in energy production and thus contribute to embryo competence (Van Blerkom, Davis, & Alexander, 2000; Van Blerkom, 2004). Mitochondrial distribution and fluorescence intensities reflect the amount of ATP generated, which correlate with embryo developmental competence (Van Blerkom et al., 2000). The nucleus controls protein synthesis and serves as a genetic blueprint during cell replication. It has been shown that nuclear chromatin morphology correlate with embryo developmental potential (Levy, Benchaib, Cordonier, Souchier, & Guerin, 1998). Most arrested or poor quality embryos display nuclear abnormalities such as chromatin condensation and fragmentation (Levy et al.,

1998). The cytoskeleton is an interconnected system of the cells that plays an important role during fertilisation and the preimplantation period (Chankitisakul, Tharasanit, Tasripoo, & Techakumphu, 2010; McKayed & Simpson, 2013). Based on the important roles of cytoskeleton during cell cleavage, it is hypothesised that they may be correlated with the timing of the first zygotic cleavage.

A previous study reported the effect of vitrification on mitochondrial distribution, mitochondrial activity and chromatin integrity of *in vivo* which produced mouse embryos (Martino et al., 2013). They found that vitrification changed the distribution pattern of mitochondria in early stage embryos, induced chromatin damage at the morula stage and reduced mitochondrial activity at the blastocyst stage (Martino et al., 2013). These findings leave open questions on whether vitrification has different effects on the mitochondria, cytoskeleton and nuclear chromatin of EC and LC embryos.

Thus, the present research was designed to study the effects of vitrification on the mitochondria, cytoskeleton (microtubules and actin filaments) as well as nuclear chromatin based on the timing of the first zygotic cleavage.

MATERIALS AND METHODS

Embryo Sources

All procedures involved were approved by the Institutional Animal Care and Use Committee (ACUC) (ACUC-7/11) of Universiti Teknologi MARA (UiTM). A total of 30 female ICR strain mice, six to eight weeks of age, weighing 25-35 g were used as embryo donors. The females were superovulated by intraperitoneal (i.p.) injections of 5 IU of Pregnant Mare Serum Gonadotrophin (PMSG: Folligon, Intervet International B.V, Holland), followed 48 hours later, by 5 IU of Human Chorionic Gonadotropin (hCG: Chorulon, Intervet International B.V, Holland). Immediately after hCG injection, the female mice were cohabited with a male stud for copulation. After 28 hours, plugged females were euthanised by cervical dislocation, and their oviducts excised. Embryos were flushed out from the oviducts into M2 medium (Sigma, USA: M7167).

Timing of the First Sygotic Cleavage

Zygotes with 2 pronuclei and embryos at the 2-cell stage were considered to be fertilised. Only embryos that had even-sized blastomeres with no cytoplasmic fragments were collected. The embryos were then divided into two groups according to the timing of their first zygotic cleavage. Embryos at the 2-cell stage at 28-30 hours post hCG administration were categorised as EC embryos whereas the zygotes with the presence of the second polar body and two pronuclei were categorised as LC embryos. The embryo pools for the EC and LC groups comprised 48 and 45 embryos respectively. The zygotes and embryos were cultured overnight in M16 medium (Sigma, USA: M7292) supplemented with Bovine Serum Albumin (BSA) (Sigma, USA: A9418).

Vitrification

After 20 hours, embryos at the 2-cell stage were vitrified using the EFS20/40 vitrification method modified from Mochida, Hasegawa, Taguma, Yoshiki and Ogura (2011). The method uses EFS20 as an equilibration solution, and EFS40 as a vitrification solution. The EFS20 contained M2 medium with 20% v/v ethylene glycol, 24% w/v ficoll 70 and 0.4 M sucrose, while EFS40 contained M2 medium with 40% v/v ethylene glycol, 18% w/v ficoll 70 and 0.3 M sucrose. After vitrification, the embryos were warmed to room temperature and then cultured in M16 for 10 minutes before staining.

Embryo Fixation

Vitrified embryos were fixed with 4% paraformaldehyde (PFA, Sigma Aldrich: P6148) in Phosphate Buffer Saline (PBS) (Sigma, USA: P4417) at room temperature, until further processing. Following fixation, the embryos were washed in PBS for 30 minutes and immunofluorescent stained through serial incubations in dyes.

Immunofluorescence Staining

Embryos were first incubated with 4', 6'-diamino-2-phenylindole dihydrochloride (DAPI) (Molecular Probes, Life Technologies, USA: D3571) for 30 minutes to stain for DNA chromatin. Then, embryos were permeabilized with 0.1% Triton X-100 (Sigma, USA: X100) in Phosphate Buffer Saline (PBS) (Sigma, USA: P4417). After a 10-minute incubation in 0.1% Triton X-100, mitochondria were incubated with MitoTracker Red Probe [MitoTracker® Red (Invitrogen, USA: M7513)] for 40 minutes. The embryos were then washed twice with PBS for 10 minutes to remove excess MitoTracker from the cytoplasm. Finally, the embryos were counterstained with DAPI for 30 minutes.

To visualise the abundance and distribution of actin filaments and microtubules, embryos were first incubated with DAPI and permeabilized with 0.1% Triton X-100 in PBS as described above. Actin filaments were labelled with Alexa Fluor 635 Phalloidin (Molecular Probes, Life Technologies, USA: A34054) while microtubules structure were labelled with Monoclonal anti-α-Tubulin conjugate with FITC (Sigma, USA: F2168) in PBS containing 1% BSA, for one hour. The embryos were then washed twice with PBS for 10 minutes. Finally, the embryos were counterstained with DAPI for 30 minutes.

The labelled embryos were mounted on glass microscope slides with an antifade medium (Pro Long Gold Antifading Agent) (Molecular Probes, Life Technologies, USA: P36934), and sealed under a coverslip. All slides were stored in the dark at 4°C prior to processing and imaging. Fluorescence signals were observed under a Confocal Laser Scanning Microscope (CLSM) (Leica TCS SP5 AOBS, Germany). The distribution pattern of actin filaments, microtubules, nuclear chromatin and mitochondria of vitrified EC and LC embryos were observed and recorded.

Quantification of Fluorescent Images

A software known as LAS AF Lite version 2.6 (Leica Microsystems CMS GmbH, Wetzlar, Germany) was used to quantify the fluorescence intensity of mitochondria, actin filaments, microtubules and nuclear chromatin in the immunofluorest images. Fluorescent intensities of mitochondria, actin filaments and microtubules were measured by manually outlining the blastomeres. Fluorescent intensities of nuclei were measured by manually outlining nuclei. Average fluorescent intensities per unit area within the region of interest (ROI) in the immunofluorescence images were measured after background subtraction.

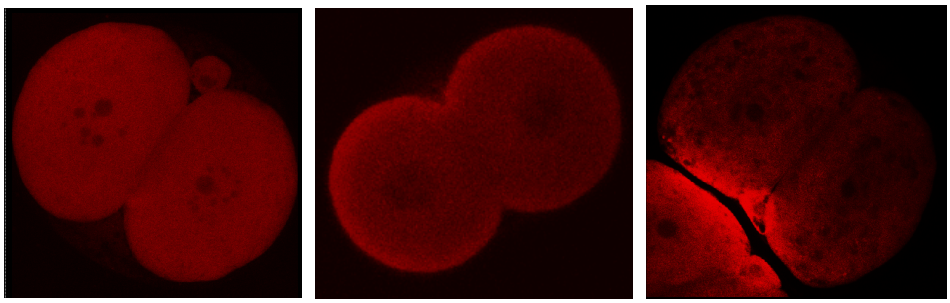
Statistical Analysis

Statistical analysis was performed using the SPSS software for Windows version 19.0.1 (Statistical Package for Social Sciences, Inc., USA). All data were presented as the means ± standard deviation (SD). Independent T-test was performed to analyse differences in - immunofluorescent intensities among vitrified EC and LC embryos. Differences were considered significant when P-values were less than 0.05.

RESULTS

Mitochondria of Vitrified Embryos

Mitochondria of 48 vitrified EC and 45 LC embryos were observed at the 2-cell stage. Mitochondria of EC embryos were seen to be homogenously distributed in the cytoplasm of blastomeres (Figure 1A). Conversely, mitochondria of LC embryos were concentrated at the pericortical region (Figure 1B). There was generally no aggregation /clustering of mitochondria in EC and LC embryos, except in one LC embryo (representing 2.2% of samples) (Figure 1C). Most vitrified EC and LC embryos (83% and 82% respectively) showed symmetrical mitochondrial distribution patterns (Table 1). Mitochondrial fluorescent intensities of EC embryos were significantly higher compared to LC embryos (39.22 ± 12.50 vs 35.42 ± 14.61 pixel) ($p<0.001$) (Figure 2).



(a) (b) (c)

Figure 1. Vitrified embryos

There are three types of vitrified embryos in the figure above. Figure 1(a) shows vitrified EC embryos, where mitochondria is distributed homogenously in the cytoplasmic region of blastomeres with higher fluorescence intensities. Figure 1(b) shows vitrified LC embryos where mitochondria is distributed homogenously in the cytoplasmic region of blastomeres with comparatively lower fluorescence intensities. Figure 1(c) is vitrified LC embryos. Here, mitochondria is distributed in cluster in the cytoplasmic region of blastomeres.

Table 1
Percentage of symmetrical mitochondrial distribution in blastomeres of vitrified EC and LC embryos

Group	Number of embryos	Percentage of embryos (%)	
		Symmetry	Asymmetry
EC embryos	48	40/48 (83%)	8/48 (17%)
LC embryos	45	37/45 (82%)	8/45 (18%)

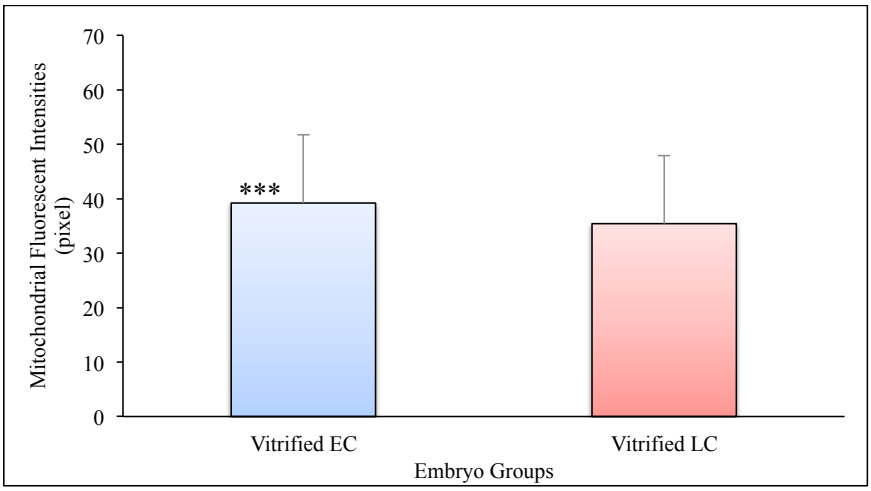
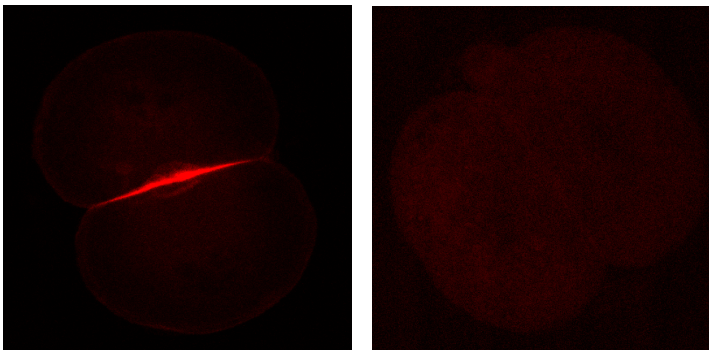


Figure 2. Mitochondrial Fluorescent Intensities in Vitrified EC vs LC Embryos (Mean ± SD)
*** vitrified EC versus LC embryos ($p<0.001$)

Cytoskeleton of Vitrified Embryos

A total of 44 vitrified EC embryos and 41 vitrified LC embryos at the 2-cell stage were processed for cytoskeletal analyses. Visual observation on cytoskeletal distributions revealed that actin filament of vitrified EC and vitrified LC embryos were mostly located at the plasma cell membrane. Vitrified LC embryos were seen to have a relatively lower abundance of actin. Generally, actin filaments of EC embryos were concentrated at the intercellular space (Figure 3).



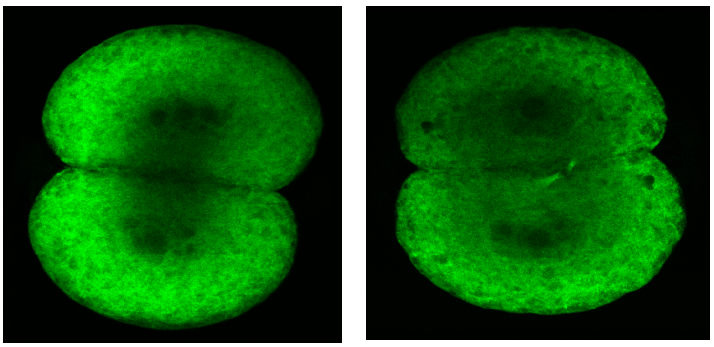
(a) (b)

Figure 3. (a) Vitrified EC embryo; and (b) vitrified LC embryo stained with antibody to actin (Alexa Fluor 635-red)

Actin of vitrified EC and vitrified LC embryos were mostly located at the plasma cell membrane. Generally, actin of vitrified EC embryos was concentrated at the intercellular space, while actin of vitrified LC embryos had a relative weak cytoplasmic background.

Tubulin of EC and LC embryos were uniformly distributed in the cytoplasm of blastomeres. However, less tubulin was seen at cell contact areas. Figure 4 illustrates localisation of tubulin in EC and LC embryos. The cytoplasm of both EC and LC embryos was brightly stained for tubulin, especially in apical and central regions. Less fluorescence was observed in the basal region.

Table 2 shows fluorescent intensities of actin and tubulin in EC and LC embryos. Vitrified EC embryos had significantly higher actin fluorescence intensities compared to LC embryos (11.43 ± 5.44 vs 5.23 ± 2.20 pixel) ($p<0.001$). No significant difference was found between tubulin fluorescent intensities of EC and LC embryos.



(a) (b)

Figure 4. (a) Vitrified EC embryo; and (b) Vitrified LC embryo

Both the vitrified EC embryo and vitrified LC embryo were brightly stained with antibody to tubulin (Monoclonal anti α-Tubulin conjugate with FITC - green). The distributions of tubulin in vitrified EC embryos were similar to vitrified LC embryos. They were distributed homogenously in the cytoplasmic region of blastomeres. Confocal images showed that the cytoplasm of the vitrified EC and vitrified LC embryos were strongly stained with anti α-tubulin (green stain).

Table 2
Mean fluorescent intensities of actin and tubulin in vitrified EC and LC embryos (Mean ± SD)

Structure	Mean ± SD (pixel)		P value
	EC embryos	LC embryos	
Actin	11.43*** ± 5.44	5.23 ± 2.20	<0.001
Tubulin	29.77 ± 7.66	23.09 ± 8.38	0.084

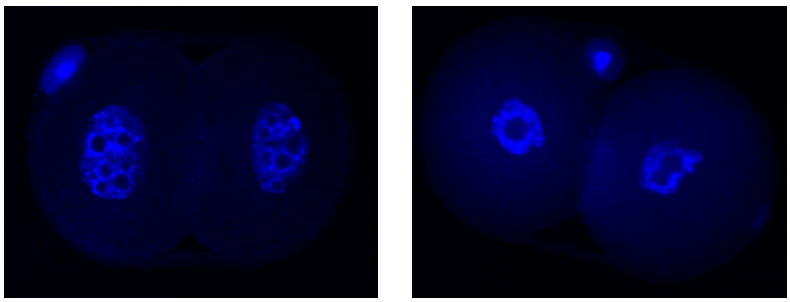
*** vitrified EC versus LC embryos ($p < 0.001$)

Nuclear Chromatin of Vitrified Embryos

A total of 52 vitrified EC embryos and 40 vitrified LC embryos at the 2-cell stage were stained to study the impact of vitrification on nuclear chromatin. Nuclei of vitrified EC and LC embryos were centrally located in each blastomere (Figure 5). There was no significant difference between nuclear fluorescent intensities in vitrified EC and LC embryos (Table 3).

Table 3
Mean fluorescent intensities of nuclear chromatin of vitrified EC and LC Embryos (Mean ± SD)

Structure	Mean ± SD (pixel)		P value
	EC embryos	LC embryos	
Vitrified embryos	204.61 ± 41.66	213.15 ± 37.43	0.337



(a) (b)

Figure 5. (a) Vitrified EC embryo; and (b) vitrified LC embryo stained with antibody to chromatin (DAPI - blue). Nuclei of vitrified EC and vitrified LC embryos were centrally located with no fragmentation

DISCUSSION

This study examined the impact of vitrification on mitochondrial distribution, cytoskeletal organisation and nuclear chromatin configurations of mouse embryos based on timing of the first zygotic cleavage. The visualisation of morphology and quantification of immunofluoresence signals provide greater understanding of the differences controlling developmental competence in EC and LC embryos, after vitrification.

Mitochondria are important for energy production, and therefore indicate the quality and developmental potential of embryos. It has been shown that intracellular localisation of mitochondria is essential for successful development of embryos (Matsumoto, Shoji, Umezu, & Sato, 1998; Van Blerkom et al., 2000). In the present study, mitochondria of vitrified EC embryos were found to be homogenously distributed in the cytoplasm of blastomeres. Previous studies showed the same mitochondrial distribution pattern in inbred blocking strain and hybrid nonblocking strain mouse and rat embryos (Bavister & Squirrell, 2000). However, in outbred blocking strain mouse (Bavister & Squirrell, 2000; Bogolyubova, 2005), human (Van Blerkom et al., 2000) and hamster (Bavister & Squirrell, 2000; Kabashima, Matsuzaki, & Suzuki, 2007) embryos, mitochondria were concentrated at the perinuclear region. It has been proposed that the perinuclear mitochondrial localisation serves to supply energy for intranuclear processes and to protect nuclear structure against oxidative stress (Bavister and Squirrell, 2000; Ramalho-Santos, et al., 2009). In mouse embryos blocked at the two-cell stage or arrested embryos, no perinuclear concentration of mitochondria was observed. These mitochondria were found to aggregate into clusters (Bogolyubova, 2005).

In the present study, mitochondria of vitrified LC embryos were concentrated at the cortical region of blastomeres. These localisation patterns were also reported in a previous study on arrested mouse embryos (Muggleton-Harris & Brown, 1988). Localisation of mitochondria at the cortical region may be related to vitrification damage. This pattern of localisation may reduce energy supply to the nuclear region, thus increasing the risk of embryo arrest after subsequent culture. Homogenous distribution of mitochondria in the cytoplasm of vitrified EC embryos may be due to better recovery after vitrification. Mitochondria localised in the cortical region may be involved in the recovery process after vitrification. In other words, vitrified EC embryos may have good relocalisation of mitochondria to adjust to changes during vitrification. Generally, there was no aggregation of mitochondria in vitrified EC and vitrified LC embryos. Aggregation of mitochondria was only observed in one embryo, which increased the risk of its developmental arrest, as reported by a previous study (Bogolyubova, 2005).

In the present study, most mitochondria were distributed symmetrically between both blastomeres of vitrified EC and LC embryos. Previous study showed that embryos with unsymmetrical mitochondrial distribution between blastomeres remained undivided and often degenerated after subsequent culture (Van Blerkom et al., 2000). Observation on confocal images in the present study also revealed higher mitochondrial fluorescence signals in vitrified EC embryos compared to vitrified LC embryos. The signals differences were also supported by quantification analysis which showed significantly higher intensities of mitochondria in vitrified EC embryos compared to vitrified LC embryos. This indicates higher energy supply

for vitrified EC embryos to promote better survivability and developmental viability after vitrification, as reported earlier (Jusof et al., 2015).

Actin filaments of vitrified EC and LC embryos were primarily located at the cell membrane or in the cortical region of blastomeres, resulting in the presence of a ring-like fluorescence around each blastomere. This observation is largely in agreement with the results from previous studies which found the location of actin just beneath the cell membrane or in the cortical layer of blastomeres (Gallicano, 2001; Kabashima et al., 2007; Lehtonen & Badley, 1980). Although the localisation patterns of actin were similar in vitrified EC and LC embryos, higher signals of Alexa Flour 635 were detected at the intercellular contact points of EC embryos. Conversely, vitrified LC embryos showed a thinner layer of actin at the cortical layer of blastomeres and also at the intercellular contact points. The higher signals of actin in cortical region of vitrified EC embryos may contribute to the higher cleavage speed in vitrified EC embryos because actin filaments play an important role in the contractile system during the cytokinesis of blastomeres (Gallicano, 2001). During cytokinesis, actin filaments that are located at the pericellular space contract, tighten and pinch the cell into two (Nelson & Cox, 2008). For that reason, actin microfilaments are concentrated in the intercellular space of dividing cells (Chankitisakul et al., 2010). Actin quality and distribution is important because it correlates with embryo viability. A normal actin content and distribution is required for embryos to undergo normal cell division (Zijlstra et al., 2008).

Actin filament distribution differences were studied by Matsumoto et al. (1998). The authors compared distribution differences in non-blocked and blocked 2-cell stage rat embryos. In non-blocked embryos, they found that actin filaments were distributed adjacent to nuclei and along the cell membrane. However, in embryos blocked at the two-cell stage, actin filaments formed granules and were dispersed in the cytoplasm. Another study found actin abnormalities in embryos arrested at early stages of development and in cryopreserved embryos (Levy et al., 1998). Another study found a significant relationship between the osmotic levels and the chances of blastocysts exhibiting disrupted cellular actin filaments (Men, Agca, Mullen, Critser, & Critser, 2005). In the present study however, no actin filaments abnormalities were detected either in vitrified EC or LC embryos.

The distribution of actin filaments are clearly related to their function. Actin filaments provide support and structure to cell membrane. They regulate cell shape by interacting with integrin proteins at the cell membrane (Calderwood, Shattil, & Ginsberg, 2000). The concentration of actin filaments along the cell membrane in both vitrified EC and LC embryos in this study may therefore be attributed to their role of regulating cell shape via interaction with integrins.

Quantification of immunofluorescence images from the present study showed significantly higher actin filaments intensities in vitrified EC embryos compared to vitrified LC embryos. Higher intensities of actin in vitrified EC embryos reflect higher abundance of actin filaments, thus conferring vitrified EC embryos with higher developmental viability compared to vitrified LC embryos (Isom et al., 2012; Lechniak et al., 2008; Lundin et al., 2001; Nielsen & Ali, 2010). Higher abundance of actin may also contribute to the maintenance of embryo shape after vitrification, thus increasing the survivability of vitrified ECs compared to LC embryos.

Another component of the cytoskeleton which demonstrates many important roles in cellular processes of preimplantation embryos are microtubules. Microtubules are involved in cell motility, cell division, control of cell shape and cytoplasmic organisation (De Brabander, 1982). They also organise the distribution of various cytoplasmic organelles including mitochondria (Heggeness, Simon, & Singer, 1978). While the function of microtubules are well known, little information is available regarding their roles during vitrification.

The distribution of microtubules is related to their function in a variety of cellular processes (Kavallaris, 2010). The present study found that microtubules of vitrified EC and LC embryos were distributed homogenously in the cytoplasm of blastomeres, especially in apical and central regions. In congruence, previous studies also found homogenous distribution of microtubules in the cytoplasm of developing two-cell stage mouse embryos (Kan et al., 2011; Matsumoto et al., 1998) with the highest density in apical cytoplasm (Van Blerkom et al., 2000). Microtubules were also found around nuclei as documented by previous studies (Kabashima et al., 2007; Van Blerkom et al., 2000), with a similar pattern in both vitrified EC and LC embryos. The present study also found less microtubules in the basal region or in the intercellular apposition of vitrified EC and LC embryos. This is in agreement with a previous study which found low abundance of microtubules in the intercellular contact of newly divided blastomeres (Van Blerkom et al., 2000). Another study however, reported a high abundance of microtubules in the area, with wide intercellular contact (Lehtonen & Badley, 1980).

In a study conducted to compare the distribution pattern in normal developing embryos and embryos exhibiting two-cell block by Matsumoto et al. (1998), the researchers found thicker fibrous microtubules which distributed as rude meshwork structures in the cytoplasm of embryos exhibiting the two-cell block. Another study found low density of microtubules without perinuclear network in blocking two-cell embryos (Neganova, Sekirina, & Eichenlaub-Ritter, 2000). The present study found microtubules to be distributed homogenously in the cytoplasmic region of both vitrified EC and LC embryos. The nucleus is the largest organelle in the cell. The main function of the nucleus is to govern gene expression and facilitate DNA replication. Fluorescence microscopy of nuclear DNA is useful for the determination of exact functional stages of early embryo development (Mori, Hashimoto, & Hoshino, 1988). The rate of cell division, metabolism and development correlate with nuclear DNA content. Nuclear fluorescent intensity in confocal images is directly proportional to DNA content of the cells (Ljosa & Carpenter, 2009).

Vitrification was reported to increase DNA fragmentation and reduce developmental viability (Park et al., 2006). The present study showed that there is no significant difference between nuclear fluorescent intensity of vitrified EC embryos and LC embryos. The present study also showed the normal nuclei localisation and morphology with no fragmentation or chromatin condensation in both vitrified EC and LC embryos. A previous study reported that nuclear abnormalities such as chromatin condensation and fragmentation were found only in arrested embryos (Levy et al., 1998). Observation showed that mouse embryos were relatively tolerant to vitrification by EFS20/40 method. The recovery process was sufficient to allow for further development.

CONCLUSION

It can be concluded therefore that EC embryos are more cryo-tolerant due to higher densities of mitochondria and actin filaments which appear to result in more efficient cell division, and therefore, greater developmental competence. Thus, selection of embryos for IVF procedure should be made based on timing of the first zygotic cleavage.

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