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Vitrification of Blastocyst Murine Embryos Affects PI3K Pathway by Modulating the Expression of XIAP and S6K1 Proteins

Mohd Fazirul, M.[1], Sharaniza, A. R.[2], Norhazlin, J. M. Y.[1,3], Wan Hafizah, W. J.[1,4], Razif, D.[1,5], Froemming, G. R. A.[2], Agarwal A.[6], Mastura, A. M.[1] and Nor Ashikin, M. N. K.[1]*

[1]Maternofetal and Embryo Research Group (MatE), Faculty of Medicine, Universiti Teknologi MARA (UiTM) Selangor, Sungai Buloh Campus, 47000 Sungai Buloh, Selangor, Malaysia
[2]Faculty of Medicine, Universiti Teknologi MARA (UiTM) Selangor, Sungai Buloh Campus, 47000 Sungai Buloh, Selangor, Malaysia
[3]Faculty of Applied Sciences, Universiti Teknologi MARA (UiTM) Perak, Tapah Campus, 35400 Tapah Road, Tapah, Perak, Malaysia
[4]Faculty of Pharmacy and Health Sciences, Universiti Kuala Lumpur Royal College of Medicine Perak (UniKL RCMP), 30100 Ipoh, Perak, Malaysia
[5]Faculty of Health Sciences, Universiti Teknologi MARA (UiTM) Selangor, Puncak Alam Campus, 42300 Puncak Alam, Selangor, Malaysia
[6]Faculty of Medicine, Northern Border University, Kingdom of Saudi Arabia

ABSTRACT

Cryopreservation by vitrification has been widely used in Assisted Reproductive Technology (ART) to preserve embryos for an extended period of time. However, the effect of vitrification on development of the embryos is lacking. Therefore, understanding on vitrification effects on embryonic proteins, especially those involved in preimplantation development is crucial to provide high quality embryos for further usage. In this study, XIAP and S6K1 protein expressions following vitrification was investigated, since they have been implicated in diverse cellular processes including cell growth, migration, proliferation, differentiation, survival and development of preimplantation embryos via the PI3K pathway. Embryos were obtained from superovulated female ICR mice which were mated with fertile males. The embryos were harvested at the 2-cell stage and cultured until blastocyst stage. Blastocysts were then vitrified in ESF40 cryoprotectant. Western blot was carried out to determine the expression of XIAP and S6K1 proteins. The results showed the expression of XIAP and S6K1 significantly decreased in vitrified

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E-mail addresses:
mfazirul@mail.com (Mohd Fazirul, M.),
sharaniza_abrahim@salam.uitm.edu.my (Sharaniza, A. R.),
norhazlin9590@perak.uitm.edu.my (Norhazlin, J. M. Y.),
whafizah@unikl.edu.my (Wan Hafizah, W. J.),
razifdasiman@salam.uitm.edu.my (Razif, D.),
gabriele@salam.uitm.edu.my (Froemming, G. R. A.),
dranshoo3@gmail.com (Agarwal, A.),
mastura.malek78@gmail.com (Mastura, A. M.),
noras011@salam.uitm.edu.my (Nor Ashikin, M. N. K.)
*Corresponding Author

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blastocyst compared to the control. This indicates that blastocyst vitrification may impact developmental competence through the activation of apoptotic pathways.

Keywords: Blastocyst, cryopreservation, embryo, PI3K, vitrification

INTRODUCTION

Studies of mammalian embryo development *in vitro*, especially in mice have provided insights into understanding the fundamental biological processes in early preimplantation development. This has enabled researchers to further understand the molecular basis of embryonic cellular differentiation and improve the quality of embryos produced in Assisted Reproductive Technologies (ART).

It is a common practice in ART today to limit transfer of embryos to maximum of two blastocysts at a time. Therefore, efficient cryopreservation method for surplus blastocyst preservation is necessary to avoid waste and for future usage (Cutting et al., 2008; Sunde, 2007). Recent studies have favoured cryopreservation by vitrification, a technique which involves the solidification of water or water-based solutions into a glass-like amorphous liquid state (Fahy, MacFarlane, Angell, & Meryman, 1984; Rienzi et al., 2017; Vajta, Cobo, Conceicao, & Yovich, 2009) as it is quick and efficient in maintaining viability of embryos.

However, the effect of vitrification on the intracellular signalling pathway is still poorly understood. Understanding of the vitrification effects on the embryos' biological processes and their regulation is important since their development depends on the signal generated by growth factors which are present in the maternal environment. These growth factors are known to regulate cell proliferation and differentiation during development of preimplantation embryos (Raff, 1992; Weil et al., 1996). A previous study by Dardik, Smith and Schultz (1992) has shown that embryos express many receptors for the ligands present in the maternal tract and those synthesised by the embryo itself.

The regulation of embryonic development during fertilisation and implantation is crucial for mammalian reproduction. Cell death occurs during preimplantation embryogenesis. Apoptosis may be involved in the embryonic arrest, producing cytoplasmic fragments. Most cells are programmed for apoptosis, with their protein components expressed and associated with inhibitors. Thus, any blockage of inhibitor synthesis will induce apoptosis. Gene expression will trigger depending on external factors, to either promote or inhibit cell death (Vinatier, Dufour, & Subtil, 1996). Deveraux et al. (1999) discovered a protein family of apoptosis inhibitor proteins (IAPs) which plays an important role in the inhibition of cell signalling apoptosis. X-linked IAP (XIAP) contains amino terminal baculoviral inhibitor of apoptosis repeat (BIR) domains and a carboxy terminal RING zinc finger (Duckett et al., 1996; Listen et al., 1996; Rothe, Pan, Henzel, Ayres, & Goeddel, 1995; Uren, Pakusch, Hawkins, Puls, & Vaux, 1996) which leads to cell death suppression when induced by various apoptotic stimuli including TNF, Fas, growth factor/serum withdrawal, chemotherapeutic agents (etoposide, actinomycin D, taxol), menadione, and UV radiation (Ambrosini, Adida, & Altieri, 1997; Duckett et al., 1996; Li et al., 1998; Listen et al., 1996). Thus, XIAP appeared to be the most potent inhibitor

of caspases, a property that is most evident with its second BIR domain (BIR 2) (Takahashi et al., 1998). It can act as an inhibitor of apoptosis in a variety of systems.

The mammalian target of rapamycin (mTOR) integrates many cellular signals that coordinate cell growth and division in response to growth factors, nutrients and energy status of cell (Bozulic & Hemmings, 2009). mTOR is implicated in various human diseases such as cancer, diabetes and cardiovascular disease (Goberdhan & Boyd, 2009; Sabatini, 2006; Strimpakos, Karapanagiotou, Saif, & Syrigos, 2009). The predetermined coordinated cell growth is greatly influenced by mTOR downstream effector such as S6 kinase 1 (S6K1), as reported previously in studies in mice (Um et al., 2006). The ability of mTOR to phosphorylate and activate S6K1 depends on three associated protein type: (1) rapamycin-sensitive adaptor protein of mTOR (raptor); (2) the G protein β-subunit-like protein (GβL); and (3) the proline-rich protein kinase B substrate 40 kDa (PRAS40). S6K1 is an important factor as a downstream effector of mTORC1 in several cellular processes, including transcription, translation, autophagy, insulin resistance and tumorigenesis in regulating cell growth, metabolism and the oncogenic phenotype (Wullschleger, Loewith, & Hall, 2006).

These are the key factors for embryonic development and differentiation into the respective stages of preimplantation embryo. Reductions of these proteins could lead to serious consequences in early embryogenesis. Consequences such as decreased proliferation and survival of the embryos depend on protein-protein intracellular interaction. Although the effect of vitrification in oocytes and embryos has been reported (Chaves et al., 2017; Lavara, Baselga, Marco-Jiménez, & Vicente, 2015; Shirazi et al., 2016; Zhou et al., 2016), it remains unknown how cryopreservation modulates the expression of XIAP and S6K1proteins, which play significant roles in the preimplantation development.

MATERIALS AND METHOD

Animal Treatment

All procedures on the animals were approved by UiTM Animal Care & Use Committee (Approval code ACUC- 7/11). Female ICR mice (8 to 10-weeks old), of 35-40 g were used as embryo donors. The mice were housed in polyurethane cages at 22±2°C, in a controlled light environment (12 hours light, 12 hours darkness) at Laboratory Animal Care Unit (LACU), Faculty of Medicine, UiTM and provided with water and standard rodents chow pellets *ad libitum*. For superovulation, the female mice were given 10 IU of pregnant mare serum gonadotrophin (PMSG), followed by 10 IU human chorionic gonadotropin (hCG) intraperitoneally, 48 hours apart. Females were then cohabited with fertile ICR males at a ratio of 1:1. Plugged embryo donors were euthanised at 23-25 hours post-HCG, by cervical dislocation, for the collection of embryos. Fallopian tubes were excised and embryos were flushed using M2 medium, under a dissecting microscope (Leica Zoom 2000, Japan). Two pronuclear-stage (2PN) embryos were cultured at 37°C with 5% CO_2 in a humidified incubator until they developed into blastocysts.

Vitrification Protocol

Blastocysts were collected and transferred to M2 medium supplemented with 5mg/ml bovine serum albumin (BSA) and kept at room temperature. The straw for vitrification was loaded with 30 µL of EFS40 solution. A total of 10 blastocysts were aspirated into the straws, with a minimal volume of M2 medium. The straws were then sealed and their contents left to equilibrate for one minute. The straws were then placed on liquid nitrogen vapour (-180°C) for five minutes before being immersed into liquid nitrogen.

Warming Protocol

Blastocyst were warmed by holding straws on air at 22 ± 2°C for 10 seconds, followed by placing them in a water bath at 37°C for 20 seconds. Straw contents were then expelled into 0.5 M sucrose, and left there for two minutes. The blastocysts were then washed with M2 medium for two minutes followed by morphological evaluation. Blastocysts of excellent, good, and fair quality were considered to have survived. The surviving blastocysts were then transferred to KSOM medium before being subjected to western blot.

Protein Extraction and Western Blot

Pooled blastocysts were lysed using the Mammalian Protein Extraction Reagent (M-PER) (Thermo Fisher Scientific, USA). A total of 100 µL of M-PER Reagent was added into 0.5 ml microcentrifuge tubes. One microlitre of Halt Protease Inhibitor Cocktail, EDTA free (Catalogue no: 87785, Thermo Fisher Scientific, USA) was added to per 100 µL of lysis buffer. The blastocyst was then gently mixed for five minutes and centrifuged at 4°C with 17,000 rpm for 10 minutes. The pellet was discarded and the supernatant was collected and stored at -80°C until further use. Protein concentration was measured using Nanodrop ND-1000 (Thermo Fisher Scientific, USA) followed by Bradford Assay Kit (Bio-Rad Laboratories, Hercules, California) for validation of protein concentration measurement. Protein samples (25 µg) were resolved by sodium dodecyl sulfate 12.5% gradient gel electrophoresis and then transferred to nitrocellulose membranes by electrophoretic blotting (Thermo Scientific Pierce, USA). Blots were then incubated with the primary antibodies at 4°C overnight. After washing, membranes were incubated for two hours with anti-mouse or anti-rabbit horseradish peroxidase-conjugated IgG (1:1000). Immunoreactivity was detected using an enhanced SuperSignal West Pico Chemiluminescence reaction (Thermo Scientific, Pierce, USA). Densitometric analysis employed Thermo Scientific myImage Analysis Software (Thermo Scientific, Pierce, USA). The following primary antibodies (Abcam, England) and dilutions were used: anti-S6K1 (ab32529, 1:100), anti-XIAP (ab28151, 1:100), and anti-b-actin (1:10000 dilution; Sigma–Aldrich).

Statistical Analysis

Data were analysed using the SPSS package programme (SPSS V. 19.0). Statistical analysis was performed using one-way ANOVA and paired sample t-test. A p value of <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Non-Vitrified Blastocyst

To investigate whether XIAP and S6K1 protein were expressed in blastocysts, western blot was performed on lysate protein of non-vitrified blastocysts. The results showed that XIAP and S6K1 protein were expressed in non-vitrified blastocyst, where the expression of XIAP proteins were significantly ($P<0.05$) higher than the S6K1 proteins (Figure 1).

Regulation of apoptosis played an important role at the blastocyst stage, since this is the last stage of preimplantation embryos where differentiation occurs. XIAP plays an important role in regulating this pathway as an inhibitor molecule. This was shown previously in which cells transfected with XIAP blocked programmed cell death in response to a variety of apoptotic stimuli (Duckett et al., 1996, 1998; Xu et al., 1999). In addition, recombinant XIAP was also demonstrated to specifically block the activity of caspases 3, 7, and 9 (Datta et al., 2000; Deveraux et al., 1998). A study by Wu, Panakanti, Li and Mahato (2010) showed that increase of XIAP expression in INS-1E cells and human islets led to decrease in the activities of caspase proteins, hence reducing apoptotic cell death.

S6K1 protein is an important downstream target of mTOR and PI3K pathways in the regulation of cell growth. They ubiquitously express serine/threonine protein kinase which phosphorylates the 40S ribosomal protein S6 in response to mitogen stimulation (Dufner & Thomas, 1999). It was clearly identified that S6K1 is activated through mTOR phosphorylation on S6K1 at Thr389, a residue whose phosphorylation is rapamycin sensitive *in vivo* (Abraham & Wiederrecht, 1996; Brown et al., 1995; Burnett, Barrow, Cohen, Snyder, & Sabatini, 1998).

The presence of XIAP in non-vitrified blastocysts in this study (Figure 1) indicated inhibition of apoptosis during preimplantation embryonic development, via internal signaling PI3K pathway. The expression of S6K1 protein at the blastocyst stage is expected as the protein plays an important role in regulating cell cycle and development.

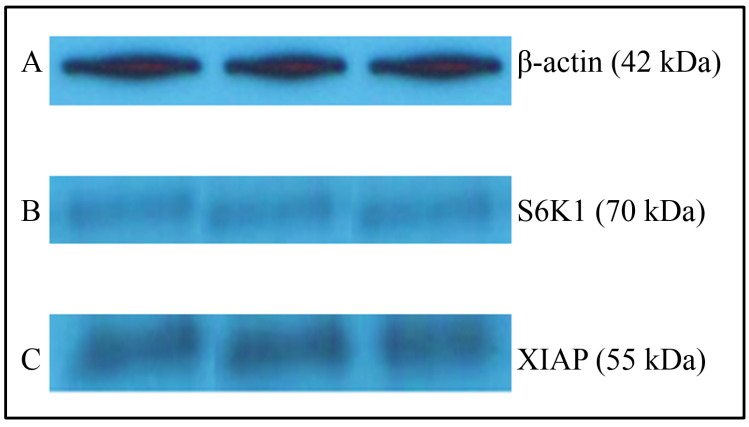

Figure 1. The expression of embryonic proteins of S6K1 and XIAP in non-vitrified blastocysts. Western blots analysis (triplicate) show expression of: (A) Positive control β-actin (42 kDa); (B) S6K1 (70 kDa); and (C) XIAP (55 kDa)

Vitrified Blastocysts

Western blot was also performed on lysate protein of vitrified blastocysts. The expression of S6K1 and XIAP proteins in vitrified blastocyst was similar to patterns in non-vitrified blastocysts (Figure 2). The expression level of XIAP was higher than S6K1. This was further confirmed by quantification of band intensity using image analysis, which showed that the expression of XIAP was significantly ($P<0.05$) higher than S6K1 (Figure 3). This shows that the expression of S6K1 is affected in vitrification, and therefore, may impair cell cycle progression of vitrified embryos. The interruption of cell cycle in vitrified blastocysts could have a negative impact on the development of the embryos. This is supported by a previous study, which showed that germline disruption of mTOR in mice caused embryonic lethality at or around implantation (Gangloff et al., 2004; Murakami et al., 2004).

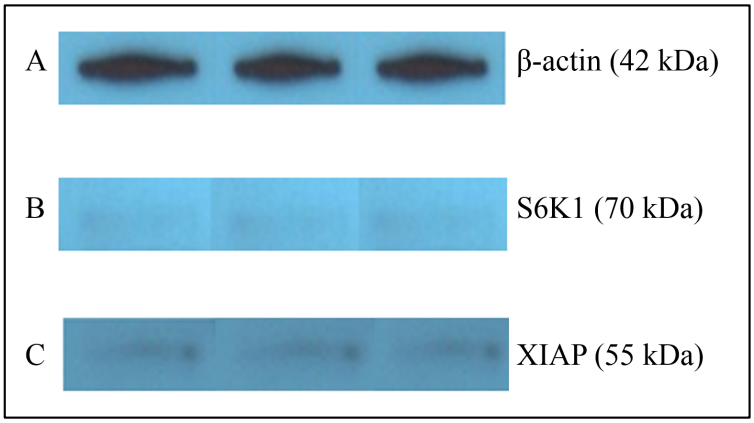

Figure 2. The expression of embryonic proteins of S6K1 and XIAP in vitrified blastocysts. Western blots analysis (triplicate) show expression of: (A) Positive control β-actin (42 kDa); (B) S6K1 (70 kDa); and (C) XIAP (55 kDa)

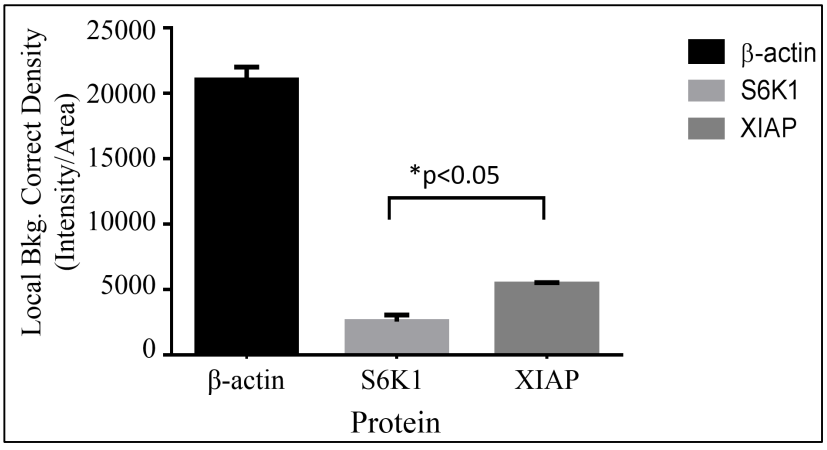

Figure 3. Protein quantifications for the expression of β-actin, S6K1 and XIAP proteins in vitrified blastocysts. The results represent the mean ± S.E.M. for three replicates. Bars indicate significant differences (one-way ANOVA, $P < 0.05$).

In addition, a comparison of the proteins expressed was made between non-vitrified and vitrified blastocyst and the data was analysed using paired t-test. From the statistical analysis, the expression of both XIAP and S6K1 was significantly higher in non-vitrified, compared to vitrified blastocysts (Figure 4). The presence of S6K1 protein in preimplantation embryos at blastocyst stages in both non-vitrified and vitrified blastocysts has been demonstrated in this study. However, the expressions of these proteins are significantly lower in vitrified embryos (Figure 4). A previous study on S6K1 knockout mice resulted in reduced size and developmental delay (Shima et al., 1998). On the other hand, S6K1 overexpression induces larger cell size (Fingar et al., 2002), and skeletal muscle cell hypertrophy (Marabita et al., 2016; Rommel et al., 2001). These studies clearly indicate the involvement of S6K1 in cellular development. In addition, Lane, Fernandez, Lamb and Thomas (1993) demonstrated that S6K1 acts as a mediator for the G1/S transition of the cell cycle. Another study showed that S6K1 catalytic activity is high during G0–G1 transition of synchronised mouse 3T3 fibroblasts, decreasing as it progressed through the cell cycle G1-M, and is activated again when cells progress from M into G1 (Edelmann, Kühne, Petritsch, & Ballou, 1996). Another study by Xu et al. (2009) showed that S6K1 was active throughout the cell cycle, with higher activity in G2 and M phases. These data suggest the importance of S6K1 in early development. In this study, it shows that the expression of S6K1 is affected in vitrified embryos.

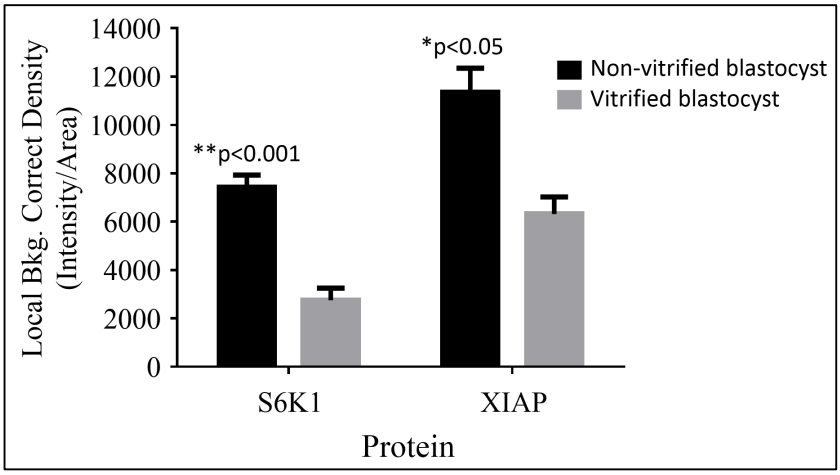

Figure 4. Protein quantifications for the expression of β-actin, S6K1 and XIAP proteins in non-vitrified and vitrified blastocyst. The results represent the mean ± S.E.M. for three replicates. Bars indicate significant differences (paired sample t-test, $P < 0.05$).

Overall, vitrified blastocyst showed a decrease in S6K1 and XIAP protein expressions compared to non-vitrified blastocysts. This indicates that vitrification negatively affected S6K1 and XIAP proteins in the PI3K pathway. The decrease in protein expression may be the result of embryos responding to significant stress and may consequently compromise embryonic development. Vitrification-warming procedures often results in cell loss and damage. It is likely that such

damage could result in alterations of autocrine secretion of growth factors and therefore heighten the impact of the post-vitrification culture environment.

During vitrification, blastocysts undergo considerable stress due to cold shock and osmotic stress (de Oliveira Leme et al., 2016) during the vitrification-warming procedure, causing morphological abnormality and functional damage. This is reflected by the lower expression of S6K1 and XIAP in vitrified blastocysts compared to non-vitrified blastocysts. Inhibition of the PI3K pathway in blastocysts may have resulted in an increase in blastocyst apoptosis and deficiencies in glucose uptake and metabolism. Further, inhibition of this pathway during this stage may result in a striking increase of pregnancy loss.

CONCLUSION

Data presented in this study showed that vitrification of murine blastocysts induced significant expression changes in the proteins involved in PI3K signalling pathways.

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