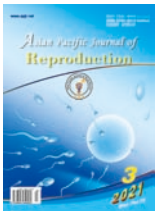

Original Article

Asian Pacific Journal of Reproduction

Journal homepage: www.apjr.net

doi: 10.4103/2305-0500.316626

Vitamin E supplementation may negatively affect preimplantation development and mitochondrial ultrastructure of vitrified murine embryos

Mimi-Sophia Sarbandi[1,2], Nor-Ashikin Mohamed Noor Khan[1]✉, Nor-Shahida Abdul Rahman[1,3], Zolkapli Eshak[4], Fathiah Abdullah[1,2], Mastura Abd Malek[1], Aqila-Akmal Mohammad Kamal[1]

[1]*Maternofetal and Embryo Research Group, Faculty of Medicine, Universiti Teknologi MARA, Selangor Branch, Sungai Buloh Campus, 47000 Sungai Buloh, Selangor, Malaysia*

[2]*Faculty of Applied Sciences, Universiti Teknologi MARA, Perak Branch, Tapah Campus, 35400 Tapah Road, Perak, Malaysia*

[3]*Faculty of Health Sciences, Universiti Teknologi MARA, Selangor Branch, Puncak Alam Campus, Bandar Puncak Alam, 42300 Kuala Selangor, Selangor, Malaysia*

[4]*Faculty of Pharmacy, Universiti Teknologi MARA, Selangor Branch, Puncak Alam Campus, Bandar Puncak Alam, 42300 Kuala Selangor, Selangor, Malaysia*

ABSTRACT

Objective: To observe the effects of vitamin E on post-vitrification preimplantation development, gross morphology as well as mitochondrial distribution and ultrastructure.

Methods: Twenty-four female C57BL/6NTac mice, aged 12-16 weeks, were randomly divided into four groups. Group A did not receive any treatment and served as the control group. Group B was treated with corn oil stripped of tocopherols and served as the vehicle group. Group C was treated with 60 mg/kg body weight of tocotrienol-rich-fraction with corn oil stripped of tocopherols. Group D was treated with 60 mg/kg body weight of alpha-tocopherol with corn oil stripped of tocopherols. All treatments were administered orally for 7 consecutive days. After superovulation and mating with fertile males, 2-cell stage embryos were harvested for vitrification. Post vitrification development *in vitro*, gross morphology and ultrastructure were compared between groups.

Results: The number of 2 and 8-cell embryo, and blastocysts in the treatment and control groups were not significantly different (P>0.05). Following vitrification, all 2-cell embryos had equal-sized blastomeres and intact zona pellucida. Mitochondrial aggregation toward the perinuclear region was seen in all of the treatment groups. Both groups C and D had vacuolated mitochondria, which was reflected in the trend of preimplantation development reduction.

Conclusions: Vitamin E supplementation of 60 mg/kg body weight does not improve the viability of healthy embryos according to this study. As a result, the most effective dose of vitamin E supplementation may be determined by the initial quality of the embryos.

KEYWORDS: Mitochondria; Preimplantation; Embryo; Vitrification; Vitamin E; Alpha-tocopherol; Tocotrienol-rich-fraction, Transmission electron microscopy

1. Introduction

In Assisted Reproductive Technology, vitrification has been an integral tool to store surplus embryos[1]. However, the procedure could lead to the production of reactive oxygen species (ROS) that compromise embryo development and implantation[2,3]. Such outcomes are likely caused by the adverse effects from the alteration of mitochondria[2,4,5], which are important organelles in the generation of energy and regulation of ROS to maintain cell homeostasis[3,6]. Many studies reported the use of antioxidants as a safe intervention to decrease ROS production[7].

Vitamin E was first discovered as an essential micronutrient for reproduction[8] and has been demonstrated for its antioxidative role[9,10] and other health benefits[11,12]. It has eight isomers which comprise a trimethyltridecyl tail with three chiral centres of α-, β-, γ-, and δ-tocopherols and unsaturated isoprenoid chain of α-, β-, γ-, and δ-tocotrienols. Vitamin E is believed to protect the plasma membrane against oxidation reactions and lipid peroxidation[11,13,14]. Several reports have also demonstrated its antioxidative effects in reducing DNA damage in cryopreserved cells[15] and improving preimplantation development of

✉To whom correspondance may be addressed. E-mail: noras011@uitm.edu.my

How to cite this article: Sarbandi MS, Nor-Ashikin MNK, Nor-Shahida AR, Eshak Z, Abdullah F, Abd Malek M, et al. Vitamin E supplementation may negatively affect preimplantation development and mitochondrial ultrastructure of vitrified murine embryos. *Asian Pac J Reprod* 2021; 10(3): 137-144.

Article history: Received: 7 December 2020; Revision: 18 February 2021; Accepted: 25 March 2021; Available online: 28 May 2021

embryos[13]. However, others have reported its adverse effect on animals and humans because of the opposing effects from different isoforms[10,16-19]. Tocotrienols have been reported to curb ROS production more efficiently than tocopherols in various *in vitro* and *in vivo* models[9,13,20]. The presence of three double bonds in their side chain grants greater mobility into cellular membranes. Such unique characteristics are present in palm tocotrienol-rich-fraction, comprising a mixture of 25% alpha-tocopherol and 75% tocotrienols. The high amount of tocotrienols in tocotrienol-rich-fraction allows greater mobility and fluidity in the cell membrane, providing specific biological and therapeutic properties[13]. This property provides tocotrienols with higher antioxidant activity compared to tocopherols[21]. A previous study on maternal supplementation of alpha-tocopherol was an observation of maternal colostrum[22]. To date, there have been no reports on the effect of maternal supplementation of tocotrienol-rich-fraction and alpha-tocopherol on mitochondrial distribution and ultrastructure in preimplantation embryos. Hence, this study aims to observe the effects of vitamin E on post-vitrification preimplantation development, gross morphology, as well as mitochondrial distribution and ultrastructure.

2. Materials and methods

2.1. Animals and maintenance

A total of 24 female mice aged 10-12 weeks old (average weight 19 g) and 12 males aged 10-12 weeks old [average weight (20-22) g] of specific pathogen-free C57BL/6NTac inbred mice were used. The mice were acclimatized for 14 days and housed in the exhaust ventilated cages with corn cob bedding (BioCOB, Germany), which was replaced every 5 days. The holding room was maintained at 12:12 hours light: dark cycle at a temperature of (20-22) ℃. The six mice in each group were housed together in the same cage lined with a corn cob. Mice were fed with high-grade commercial rodent chow (Altromin Spezialfutter GmbH & Co., Lage, Germany) and water was provided *ad libitum*.

2.2. Chemicals and standards

The alpha-tocopherol was purchased from Sigma-Aldrich (St. Louis, Missouri, USA; Cat. No: 258024), while the corn oil stripped of tocopherols was purchased from ICN Biomedicals (Ohio, USA; Cat. No: #0290141584-400). Tocotrienol-rich-fraction (Gold Tri. E™70) was purchased from Sime Darby (Kuala Lumpur, Malaysia). The percentage ratio of total content for both alpha-tocopherol and tocotrienols from tocotrienol-rich-fraction was 25%:75%. The embryo manipulation tools were purchased from Lab IVF (Kuala Lumpur, Malaysia).

2.3. Collection of 2-cell embryos

The female mice aged 12 to 16 weeks-old were treated with 60 mg/kg body weight as recommended based on the earlier studies in rodents[13,20]. The mice were divided into 4 groups, with 6 mice in each group. Group A received no treatment and served as the negative control group. Group B, which served as a positive control, was treated with the vehicle comprising corn oil stripped of tocopherols (vehicle). Group C was treated with 60 mg/kg body weight of tocotrienol-rich-fraction in corn oil stripped of tocopherols (tocotrienol-rich-fraction + vehicle). Group D was treated with 60 mg/kg body weight of alpha-tocopherol in corn oil stripped of tocopherols (alpha-tocopherol + vehicle). All treatments were administered orally for 7 consecutive days.

On day-4 and day-6 of the treatment period, the female mice were superovulated with 5 IU pregnant mare serum gonadotropin followed 48 h later by 5 IU human chorionic gonadotropin (hCG) *via* intraperitoneal injections. Immediately after the hCG injection, each female mouse was mated with one fertile male mouse of the same strain (1:1 mating ratio). Females with copulation plugs were euthanized by cervical dislocation 48 h after hCG injection to obtain 2-cell embryos. A total number of 220 embryos at the 2-cell stage from the four groups were observed.

2.4. Vitrification-warming of 2-cell embryos

Two-cell embryos were vitrified by using a two-step procedure[23] with modification. Embryos were initially equilibrated in EFS20 (equilibration solution) in base medium (PB1) of 20% (v/v) ethylene glycol, 24% w/v Ficoll 70 and 0.4 M sucrose, for 1 min and transferred to EFS40 contained PB1 medium of 40% (v/v) ethylene glycol, 18% w/v Ficoll 70 and 0.3 M sucrose for 2 min. Embryos in EFS40 were then loaded into straws and placed on cold vapour for 1 min before immersion into liquid nitrogen. After vitrification, the straws were held in the air for 10 s before being immersed in a 37 ℃ water bath for 10 s. The contents of the straws were expelled into the M2 medium (Sigma-Aldrich, USA, Cat. No: M7167) that contained the thawing solution (TS1) (0.5 M sucrose). After 5 min incubation in TS1, the embryos were transferred to a new dish of M2 medium. The embryos were sequentially transferred to the second and third M2 droplets. The embryos were then cultured *in vitro* until the blastocyst stage in M16 medium (Sigma-Aldrich, USA, Cat. No: M7292).

2.5. In vitro culture and embryo developmental assessment

The gross morphology of the 2-cell embryos was initially determined as either normal or abnormal, based on morphology[24]. Embryos that had equal size and number of blastomeres, with less than 10% fragmentation, were defined as morphologically normal.

All embryos from Groups A, B, C and D were vitrified-warmed at the 2-cell stage[23] and the recovered embryos were transferred to 50 μL droplets of M16 medium and cultured in a humidified 5% CO_2 incubator at 37 ℃. Embryo viability was assessed through observation of embryonic development until the blastocyst stage.

2.6. Transmission electron microscopy assessment

The sample was prepared according to the published protocol[25]. Eight cell-stage embryos were selected for ultrastructural assessment, as the number of blastomeres at this stage allowed for easier visualization. The 8-cell stage embryos were fixed with 2.5% glutaraldehyde overnight at 4 ℃. They were washed with three changes of cacodylate buffer for 10 min each at room temperature, followed by fixation with 2% osmium oxide for 2 h at 4 ℃. The embryos were then subjected to serial dehydration with acetone (35%, 10 min; 50%, 10 min; 75%, 10 min; 95%, 5 min; and 100%, 10 min) before being transferred into a poly-L-lysine-coated beam capsule. Agar 100 resin-acetone was added and left at room temperature for 1 h, followed by a 1:3 of resin: acetone ratio for another 2 h. The sample was then infiltrated within 100% resin overnight. Finally, the resin was replaced by freshly prepared 100% resin and was polymerized at 60 ℃ for 24-48 h. The sample was then sectioned and stained with uranyl acetate and lead citrate. The sections were then viewed under the transmission electron microscope (Tecnai G2, FEI Co., Oregon, USA) at magnification between 6 000× to 8 700×.

2.7. Statistical analysis

All statistical analyses were conducted using GraphPad Prism version 8.0.0 (GraphPad Software, San Diego, California, USA). Two-cell, eight-cell embryos and the blastocysts were scored based on qualitative assessment. Each categorical variable was described as a frequency and percentage, with between-group differences tested by the *Chi*-square test. $P<0.05$ was considered statistically significant. Descriptive analysis was carried out to assess changes in the ultrastructure of the cultured 8-cell embryos after vitrification at the 2-cell stage.

2.8. Ethics approval statement

The animal care, handling as well as experimentation was performed as per requirements of the Committee on Animal Research and Ethics (UiTM CARE), Universiti Teknologi MARA, Malaysia (approval code: ACUC/CA/01/2014).

3. Results

3.1. Developmental of vitrified 2-cell embryos

Table 1 and Figure 1 show the embryonic development after vitrification. The post-vitrification survival rates of vitrified 2-cell embryos were not significantly different between the control (100%), corn oil stripped of tocopherols (96.3%), tocotrienol-rich-fraction (89.5%), and alpha-tocopherol (93.1%) groups ($P>0.05$). No significant differences were also observed in the developing 8-cell embryos between the control (84.3%), corn oil stripped of tocopherols (80.8%), tocotrienol-rich-fraction (80.4%) and alpha-tocopherol (94.4%) groups ($P>0.05$). Similarly, the developmental rates of vitrified 2-cell embryos to the blastocyst (53.8%) stage in the corn oil stripped of tocopherols group were not significantly different when compared with the control group (74.5%), as well as that of the tocotrienol-rich-fraction group (56.9%) and alpha-tocopherol group (64.8%) ($P>0.05$). Among the treatment groups (Groups C and D), the blastocyst formation rate for the alpha-tocopherol group (Group D) was higher than the tocotrienol-rich-fraction group (Group C). However, no significant difference was observed (Table 1).

Warming resulted in blastomeres of equal size and little fragmentation in recovered 2-cell embryos. The two-cell vitrified embryos in all groups appeared morphologically normal. Figure 1 depicts the general morphology of 2-cell vitrified embryos and blastocysts. All vitrified embryos had intact zona pellucida. The morphology of normal 2-cell embryos was consistent across all vitrified groups. As shown in Figures 1 A-D, they showed reestablishment of blastomere shape and size with low fragmentation. All groups produced normal blastocysts with similar characteristics, including a tightly compacted inner cell mass and trophectoderm with many cells lining the blastocoel cavity (Figures 1E-H).

Table 1. Embryonic development after vitrification-warming at 2-cell stage.

Parameters	Group A	Group B	Group C	Group D
Embryos, *n*	51	54	57	58
Recovered 2-cell after vitrification, *n*/total (%)	51/51 (100.0)	52/54 (96.3)	51/57 (89.5)	54/58 (93.1)
Development of 8-cell stage, *n*/total (%)	43/51 (84.3)	42/52 (80.8)	41/51 (80.4)	51/54 (94.4)
Formation of blastocyst stage, *n*/total (%)	38/51 (74.5)	28/52 (53.8)	29/51 (56.9)	35/54 (64.8)

Values are presented as numbers with percentage in parentheses. Each categorical variable is described as a percentage, with between-group differences tested by the *Chi*-square test. No significance differences are observed between groups, $P>0.05$. Group A receives no treatment and serves as the normal control group; Group B receives corn oil stripped of tocopherols and serves as the vehicle group; Group C receives tocotrienol-rich-fraction plus corn oil; Group D receives alpha-tocopherol plus corn oil.

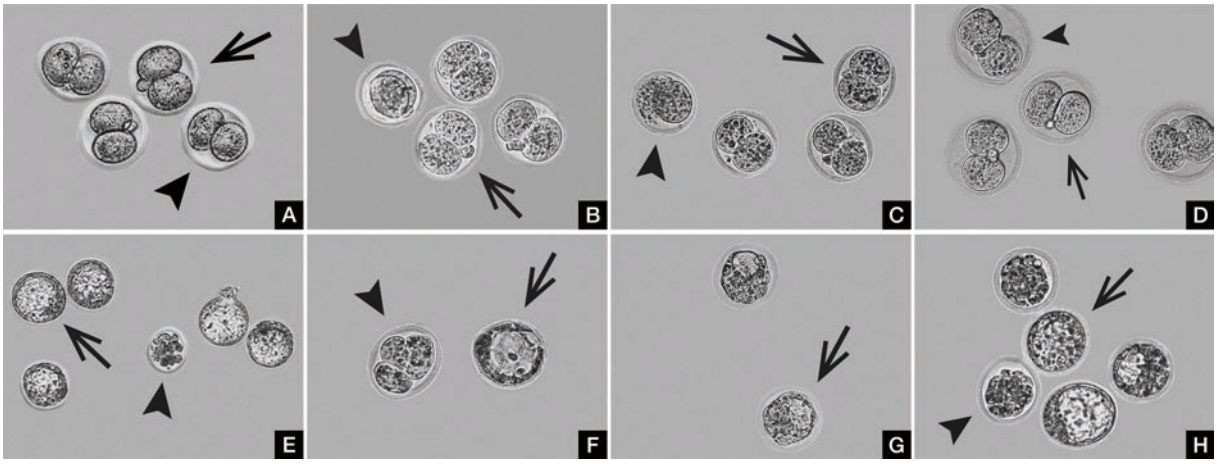

Figure 1. Photomicrograph of the vitrified 2-cell embryos developed to blastocyst stage from the C57BL/6NTac mice from (A) and (E) without treatment (control); (B) and (F) corn oil of stripped tocopherols; (C) and (G) tocotrienol-rich-fraction plus corn oil; (D) and (H) alpha-tocopherol plus corn oil groups, at magnification 200×. Arrows indicate normal morphology and arrowheads indicate abnormal morphology. The zona pellucida of all vitrified embryos are found to be intact (Figures 1A-H). The morphology of normal 2-cell embryos across all vitrified groups is similar, showing reestablishment of blastomere shape and size, with a low degree of fragmentation (Figures 1A-D). Embryos with abnormal morphology is also detected (Figures 1A & 1C). Embryos with unequal size and shape with fragmented blastomere are observed (Figures 1B, 1C & 1D). Normal blastocysts with compacted cell mass, and trophectoderm with many cells lining the blastocoel cavity (Figures 1E-H) are seen. Degenerated blastocysts are also observed (Figures 1E, 1F & 1H).

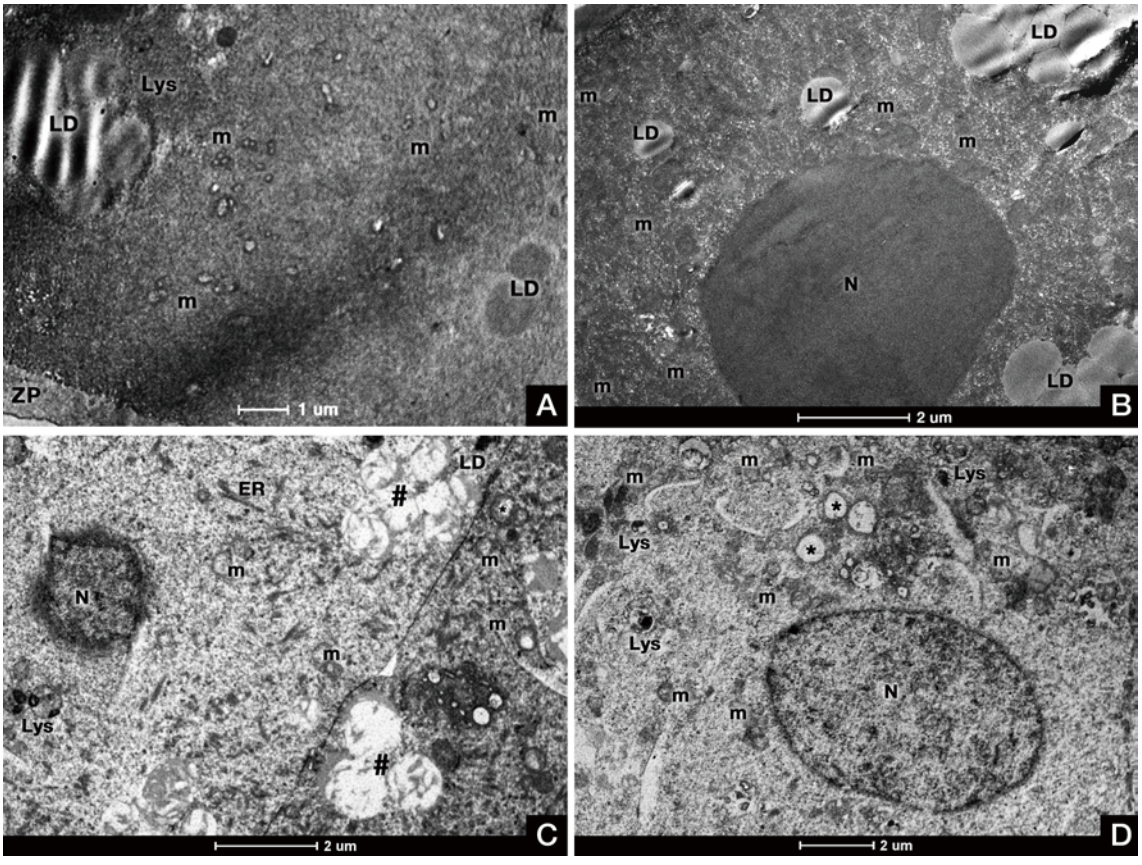

Figure 2. Ultrastructural images of 8-cell embryos developed *in vitro* from vitrified 2-cell embryos of (A) Without treatment (control) at magnification 8 200×; (B) Corn oil stripped of tocopherols group at magnification 8 700×; (C) Tocotrienol-rich-fraction + corn oil group at magnification 8 200× and (D) Alpha-tocopherol + corn oil group at magnification 6 000×. N: nucleus; m: mitochondria; Lys: lysosome; LD: lipid droplets; ZP: zona pellucida. The symbol * indicates the vacuolated mitochondria and the symbol # shows clustering of white crystalline vesicles. Note that magnification differs.

3.2. Ultrastructure assessment of vitrified embryos

Further assessment using transmission electron microscopy to investigate the ultrastructural alteration of organelles, particularly the mitochondria was carried out on *in vitro* cultured 8-cell embryos which were vitrified at the 2-cell stage. The vitrified embryos showed immature mitochondria with few peripherally localized cristae (Figure 2). Lipid droplets and mitochondria were observed

in the cytoplasm of the control group (Figure 2A). In this study, the corn oil stripped of the tocopherol group (Group B) had the most noticeable lipid droplet build-up. There was also perinuclear clustering of mitochondria and densely associated endoplasmic reticulum (Figure 2B). Vacuolated and swollen mitochondria were seen in both the tocotrienol-rich-fraction and the alpha-tocopherol groups (Figures 2C and 2D, respectively). Most mitochondria in the alpha-tocopherol group were morphologically normal and clustered. The periclustering of mitochondria was more conspicuous in the alpha-tocopherol group than the corn oil stripped of the tocopherol group. The tocotrienol-rich-fraction group, on the other hand, did not show any perinuclear clustering of mitochondria. Clear traverse ridges and clusters of ovoid-shaped mitochondria were found all over the nucleus in group D (Figure 2D). Lysosomes and other organelles were also abundant in the alpha-tocopherol group (Figure 2D). White crystalline vesicle-like structures were found in the tocotrienol-rich-fraction and alpha-tocopherol groups (Figures 2C and 2D, respectively). When compared to the alpha-tocopherol group, such structures were more evident in the tocotrienol-rich-fraction group. The ER was found dispersed in the cytoplasm in several small segments. In the tocotrienol-rich-fraction group, they were more prominent than in the alpha-tocopherol group (Figures 2C and 2D).

4. Discussion

Over the past decades, there has been an increasing trend in the application of tocotrienols as an adjunct treatment for many clinical diseases[9,11,12] and a safe intervention for other health concerns such as fertility issues[13,20]. We discovered that maternal supplementation with tocotrienol-rich-fraction resulted in normal 2-cell embryos in this study. This corresponds to the findings of our previous study[25]. Vitrification at the 2-cell stage was chosen for this study due to previous protocols' high post-warming recovery and survival rates[23,26,27]. In this study, the 2-cell embryos from all groups showed high post-warming recovery rates. Embryos that appeared morphologically normal had equal blastomeres with intact zona pellucida, as did embryos from the control group. At the 2-cell stage, all groups showed the presence of fragmented blastomeres.

Although not statistically significant, this study found that embryos from the alpha-tocopherol group had the highest percentage of 8-cell formation (94.4%) compared to the corn oil stripped tocopherols group (80.8%) and tocotrienol-rich-fraction group (80.4%). This was consistent with previous research, which found that embryos from the alpha-tocopherol group had higher preimplantation survival[14,15,25,28]. The isomer has been known to inhibit the production of free radicals due to the bioavailability of its transport proteins[11]. Except for group D, all of the treatment groups in the study show a significant pattern of slower development. We found that the corn oil group (53.8%) had a lower developmental rate to blastocyst than the control group (74.5%), as well as the tocotrienol-rich-fraction group (56.9%) and alpha-tocopherol group (64.8%). This finding does not concur with previous studies which indicated that the alpha-tocopherol group provided a better chance of survival for development *in vitro* than the tocotrienol-rich-fraction group[14,25,29,30]. Low developmental rates in the corn oil group which was stripped of tocopherols indicated that deficiency of tocopherols from vitamin E impairs preimplantation development, as reported by other studies[8,11].

This study backs up the idea that alpha-tocopherol is essential for mitochondrial metabolism balance. The mitochondria primarily determine the viability of preimplantation embryos. Asymmetrical mitochondrial distribution resulted in differential inheritance between blastomeres and reduced adenosine triphosphate generating capacity[31]. Mitochondrial insufficiency could lead to the retardation of embryonic development[5].

We also discovered that 8-cell stage embryos had immature mitochondria with peripherally localized cristae in a radial array, as previously reported[2,4]. Vitrification procedures can cause mitochondria to mediate apoptosis and overcome the imbalance caused by oxidative stress production[2,3,32]. However, some embryos were able to withstand the stress of vitrification and progress to blastocyst level.

The presence of swollen and intra-vacuolated mitochondria were observed in both the tocotrienol-rich-fraction and alpha-tocopherol groups. This finding suggests that there was a change in the cytoskeletal framework, causing cytoplasmic obstruction to mitochondria dynamics. Mitochondrial distribution changed from homogenous to perinuclear distribution when aggregation-disaggregation or active stage-specific translocation took place. It has been reported that perinuclear mitochondrial clustering could lead to the elevation of ROS accumulation around the nucleus and throughout the cell, causing cell death[30,32-34]. Therefore, the mitochondrial aggregation could have caused a decrease in the number of viable embryos in this study. Similar observations were also reported in other studies[5,26, 27].

There was no perinuclear clustering of mitochondria in the tocotrienol-rich-fraction group, as observed in the alpha-tocopherol group. This may be attributed to the distortion of cell shape, caused by clustered white crystalline vesicles. Autophagy mechanisms, which actively prevented mitochondrial clustering, may be linked to the existence of these vesicles. Previous studies indicated that regulated oxidative stress could initiate diverse cellular responses ranging from triggering signalling pathways in cell protection and coordinated mitochondrial fission and autophagy[30,33,34]. Clearance of abnormal mitochondria and cells through mitophagy protects neighbouring mitochondria and cells[35]. The mitochondria appeared

to be disorganized, suggesting that cytoplasmic degeneration had taken place. This reflects an ongoing process of necrosis. It has been documented that necrotic cell death can occur when all mitochondria in a cell are deprived of ATP due to their altered structure[30,36].

Lysosomes were also seen engulfing distorted mitochondria during the ultrastructural review. Since mitochondria are dynamic, they moved into the cell body to be repaired by fusion with another healthy mitochondrion or degraded by mitophagy[35]. Tocotrienol supplementation appeared to have resulted in mitochondrial destabilization triggered apoptosis and exacerbation of autophagy, which is consistent with a previous finding[21,33].

The tocotrienol-rich-fraction group also had small segments of endoplasmic reticulum dispersed in the cytoplasm.Fragmentation of the endoplasmic reticulum could affect its synergistic association with mitochondria and lead to ionic imbalance and inflammation[19,37]. Exposure to physical stimuli or stress from the vitrification-warming procedure or culture system could activate the endoplasmic reticulum to trigger the stress response, thereby affecting mitochondrial function[7].

Vitrification[32], drugs, and diet[38] have been shown to make mitochondria vulnerable and sensitive to stress. Tocotrienols have been shown to cause severe mitochondrial dysfunction, particularly pore formation, which releases apoptogenic proteins, resulting in energy loss and the production of reactive oxygen species (ROS)[30,33]. This could explain why the tocotrienol-rich-fraction group had a lower development rate.

Embryos in the alpha-tocopherol group also had intra-vacuolated mitochondria, densely clustered mitochondria, and short endoplasmic reticulum arrays. Despite such alterations, the alpha-tocopherol group still produced high blastocyst viability compared with other groups. This could be due to several reasons. It could be due to the bioavailability of the alpha-tocopherol transport proteins that may have inhibited the production of free radicals and prevented the further formation of oxidative stress that could affect embryo development[11,15,39,40]. While alpha-tocopherol was ineffective in blocking ROS development, the study showed that it protected against cell lysis[36]. This indicates that alpha-tocopherol may be used as an antioxidant to improve cryotolerance, as supported by other studies[29,40].

On the other hand, tocotrienols are suggested as a potent mediator connecting mitochondrial apoptosis and autophagy in response to stress[30,33]. The autophagic mechanism from these mitochondria could have released pro-apoptotic substances[14]. Such a condition has been shown to cause increased levels of prooxidants and imbalance in cells. These results indicate that the dosage of tocotrienol-rich-fraction administered impaired embryonic development to blastocyst. The presence of tocotrienols in tocotrienol-rich-fraction may have caused developmental arrest through its anti-proliferative action. The presence of alpha-tocopherol in tocotrienol-rich-fraction may have counteracted the impact of tocotrienols. Although findings from this study showed the impact of vitamin E supplementation, particularly tocotrienols, on vitrified embryos, further studies on the supplementation dosage and elucidation of redox states are necessary as variation in redox states exists between different strains in response to the application of antioxidants[41].

The purpose of this study was to assess the effect of 60 mg/kg body weight vitamin E on vitrified embryos. It was also demonstrated that supplementing with vitamin E at a dose of 60 mg/kg body weight did not improve the viability of healthy embryos. In contrast, previous studies on nicotine-treated embryos found that the same dose of vitamin E improved viability[13,20]. The high dose of vitamin E (60 mg/kg body weight) may have negatively affected the healthy embryos due to its prooxidant nature. Many antioxidants, including vitamin E have been reported to exhibit prooxidant effects because of their physical and attributes. A high level of tocopheroxyl radical from vitamin E in the cells may cause unfavorable side effects, such as increased lipid peroxidation and damage to biological constituents[42]. This was evident from the ultrastructural assessment of this study. It is a noteworthy finding which highlights that the same dose of vitamin E may have opposite effects in healthy versus stressed embryos. Therefore, serious consideration must be given when choosing the most effective dose of vitamin E.

In conclusion, this study provides evidence that maternal supplementation of vitamin E at a high dosage of 60 mg/kg body weight adversely affected preimplantation development and mitochondrial ultrastructure of vitrified murine healthy embryos. Further investigation of the dose-dependent effect of vitamin E will provide better insights into the observed effects.

Conflict interest of statement

The authors declare that there is no conflict of interest.

Acknowledgements

We wish to thank Abu Thalhah Abdul Aziz from the Institute of Medical Molecular Biotechnology (IMMB), Faculty of Medicine, Universiti Teknologi MARA Sungai Buloh Campus, Selangor Branch, for providing technical supports for this study. We further thank the staff from the Laboratory Animal Care Unit (LACU) for providing animal care and maintenance, the staff from the Imaging Unit, Faculty of Pharmacy, Universiti Teknologi MARA Puncak Alam Campus, Selangor Branch, and Electron Microscopy Lab, Department of Diagnostic Laboratory Services, Hospital Universiti Kebangsaan Malaysia (HUKM) for their electron microscopy services and support.

Funding

This research was supported financially by Universiti Teknologi MARA Grants [600-IRMI/REI/5/3(022/2019)] and [600-IRMI/MYRA 5/3/MITRA (008/2017)-2].

Authors' contributions

Nor-Ashikin Mohamed Noor Khan provided the funding. Mimi-Sophia Sarbandi and Nor-Ashikin Mohamed Noor Khan contributed to the conception and design of this study, carried out the search for the articles and drafted the manuscript. Mimi-Sophia Sarbandi, Nor-Ashikin Mohamed Noor Khan, Nor-Shahida Abdul Rahman, Zolkapli Eshak, Fathiah Abdullah, Mastura Abd Malek and Aqila-Akmal Mohammad Kamal were responsible for the analysis and interpretation of data and helped draft the manuscript. Mimi-Sophia Sarbandi wrote the manuscript. Nor-Ashikin Mohamed Noor Khan, Zolkapli Eshak and Fathiah Abdullah participated in the article screening and critically revising the manuscript. Nor-Ashikin Mohamed Noor Khan edited and approved the final manuscript. All authors have edited and approved the final manuscript.

References

[1] Vajta G. Vitrification of the oocytes and embryos of domestic animals. *Anim Reprod Sci* 2000; **60–61**: 357-364.

[2] Van Blerkom J. Mitochondrial function in the human oocyte and embryo and their role in developmental competence. *Mitochondrion* 2011; **11**: 797-813.

[3] Zorov DB, Juhaszova M, Sollott SJ. Mitochondrial reactive oxygen species (ROS) and ROS-induced ROS release. *Physiol Rev* 2014; **94**(3): 909-950.

[4] Van Blerkom J, Davis P, Alexander S. Differential mitochondrial distribution in human pronuclear embryos leads to disproportionate inheritance between blastomeres: Relationship to microtubular organization, ATP content and competence. *Hum Reprod* 2000; **15**: 2621-2633.

[5] Van Blerkom J. Mitochondria in early mammalian development. *Semin Cell Dev Biol* 2009; **20**: 354-364.

[6]Hara T, Kin A, Aoki S, Nakamura S, Shirasuna K, Kuwayama T, et al. Resveratrol enhances the clearance of mitochondrial damage by vitrification and improves the development of vitrified warmed bovine embryos. *PLoS One* 2018; **13**: e0204571.

[7]Len JS, Koh WSD, Tan SX. The roles of reactive oxygen species and antioxidants in cryopreservation. *Biosci Rep* 2019; **39**: BSR20191601. doi: 10.1042/BSR20191601.

[8]Evans HM, Bishop KS. Existence of a hitherto unknown dietary factor essential for reproduction. *Nutr Rev* 1990; **48**(4): 178-180.

[9]Aggarwal BB, Sundaram C, Prasad S, Kannappan R. Tocotrienols, the vitamin E of the 21st century: Its potential against cancer and other chronic diseases. *Biochem Pharmacol* 2010; **80:** 1613-1631.

[10]Kontush A, Finckh B, Karten B, Kohlschütter A, Beisiegel U. Antioxidant and prooxidant activity of alpha-tocopherol in human plasma and low density lipoprotein. *J Lipid Res* 1996; **37**: 1436-1448.

[11]Brigelius-Flohé R, Kelly FJ, Salonen JT, Neuzil J, Zingg JM, Azzi A. The European perspective on vitamin E: Current knowledge and future research. *Am J Clin Nutr* 2002; **76**: 703-716.

[12]Sen CK, Khanna S, Roy S. Tocotrienols in health and disease: The other half of the natural vitamin E family. *Mol Aspects Med* 2007; **28**: 692-728.

[13]Kamsani NH, Ab-Rahim S, Kamsani YS, Noor Khan NAM, Rajikin MH. Tocotrienol-rich fraction modulate the phosphoinositide 3-kinase/Akt signaling pathway genes and prevent oxidative stress in nicotine-induced preimplantation embryos. *Int J Appl Pharm* 2019; **11**: 80-85.

[14]Natarajan R, Shankar MB, Munuswamy D. Effect of alpha-tocopherol supplementation on *in vitro* maturation of sheep oocytes and *in vitro* development of preimplantation sheep embryos to the blastocyst stage. *J Assist Reprod Genet* 2010; **27**: 483-490.

[15]Hossein MS, Hashem MA, Jeong YW, Lee MS, Kim S, Kim JH, et al. Temporal effects of alpha-tocopherol and *L*-ascorbic acid on *in vitro* fertilized porcine embryo development. *Anim Reprod Sci* 2007; **100:** 107-117.

[16]Bouwstra RJ, Nielen M, Stegeman JA, Dobbelaar, Newbold JR, Jansen EHJM, et al. Vitamin E supplementation during the dry period in dairy cattle. Part I: Adverse effect on incidence of mastitis postpartum in a double-blind randomized field trial. *J Dairy Sci* 2010; **93**: 5684-5695.

[17]Brigelius-Flohé R. Adverse effects of vitamin E by induction of drug metabolism. *Genes Nutr* 2007; **2**: 249-256.

[18]Cook-Mills JM. Isoforms of vitamin E differentially regulate PKC and inflammation: A review. *J Clin Cell Immunol* 2013; **4**: 1000137.

[19]McCary CA, Abdala-Valencia H, Berdnikovs S, Cook-Mills JM. Supplemental and highly elevated tocopherol doses differentially regulate allergic inflammation: Reversibility of alpha-tocopherol and gamma-tocopherol's effects. *J Immunol* 2011; **186**: 3674-3685.

[20]Syairah MMS, Kamsani YS, Rajikin MH. An experimental examination on the effects of supplementations with palm tocotrienol-rich-fraction (TRF) and annatto Δ-tocotrienol on body weight and preimplantation embryonic development in nicotine-treated mice. *Int J Recent Technol Eng* 2019; **8**: 93-96.

[21]Rickmann M, Vaquero EC, Malagelada JR, Molero X. Tocotrienols induce apoptosis and autophagy in rat pancreatic stellate cells through the mitochondrial death pathway. *Gastroenterology* 2007; **132**: 2518-2532.

[22]Melo LR, Clemente HA, Bezerra DF, Dantas RC, Ramalho HM, Dimenstein R. Effect of maternal supplementation with vitamin E on the concentration of alpha-tocopherol in colostrum. *J Pediatr (Rio J)* 2017; **93**: 40-46.

[23]Mochida K, Hasegawa A, Taguma K, Yoshiki A, Ogura A.

Cryopreservation of mouse embryos by ethylene glycol-based vitrification. *J Vis Exp* 2011; **57**: 3155.

[24]Khalili MA, Anvari M. The effect of *in vitro* culture on cleavage rates and morphology of the *in vivo*- developed embryos in mice. *Iran J Reprod Med* 2007; **5**: 17-22.

[25]Sarbandi MS, Eshak Z, Abd Rahman NS, Abdul Aziz AT, Rajikin MH, Hashim N, et al. Palm tocotrienol-rich-fraction yields higher numbers of normal embryos whereas alpha-tocopherol produces higher preimplantation survival in murine embryos. *Malays J Fundam Appl Sci* 2018; **14**: 407-410.

[26]Martino NA, Dell'Aquila ME, Cardone RA, Somoskoi B, Lacalandra GM, Cseh S. Vitrification preserves chromatin integrity, bioenergy potential and oxidative parameters in mouse embryos. *Reprod Biol Endocrinol* 2013; **11**: 27.

[27]Ren L, Fu B, Ma H, Liu D. Effects of mechanical delipation in porcine oocytes on mitochondrial distribution, ROS activity and viability after vitrification. *Cryo Letters* 2015; **36**: 30-36.

[28]Thiyagarajan B, Valivittan K. Ameliorating effect of vitamin E on *in vitro* development of preimplantation buffalo embryos. *J Assist Reprod Genet* 2009; **26**: 217-225.

[29]Niki E. Role of vitamin E as a lipid-soluble peroxyl radical scavenger: *In vitro* and *in vivo* evidence. *Free Radic Biol Med* 2014; **66**: 3-12.

[30]Takahashi K, Loo G. Disruption of mitochondria durin tocotrienol-induced apoptosis in MDA-MB-231 human breast cancer cells. *Biochem Pharmacol* 2004; **67**: 315-324.

[31]Harvey A. Mitochondria in early development: Linking the microenvironment, metabolism and the epigenome. *Reproduction* 2019; **157** (5): R159-R179.

[32]Barbour JA, Turner N. Mitochondrial stress signaling promotes cellular adaptations. *Int J Cell Biol* 2014; **2014**: 156020.

[33]Vaquero EC, Rickmann M, Molero X. Tocotrienols: Balancing the mitochondrial crosstalk between apoptosis and autophagy. *Autophagy* 2007; **3**: 652-654.

[34]Al-Mehdi AB, Pastukh VM, Swiger BM, Reed DJ, Patel MR, Bardwell GC, et al. Perinuclear mitochondrial clustering creates an oxidant-rich nuclear domain required for hypoxia-induced transcription. *Sci Signal* 2012; **5**(231): ra47. doi: 10.1126/scisignal.2002712.

[35]Gustafsson AB, Dorn II GW. Evolving and expanding the roles of mitophagy as a homeostatic and pathogenic process. *Physiol Rev* 2019; **99**: 853-892.

[36]Nikoletopoulou V, Markaki M, Palikaras K, Tavernarakis N. Crosstalk between apoptosis, necrosis and autophagy. *Biochim Biophys Acta– Mol Cell Res* 2013; **1833**: 3448-3459.

[37]Filadi R, Theurey P, Pizzo P. The endoplasmic reticulum-mitochondria coupling in health and disease: Molecules, functions and significance. *Cell Calcium* 2017; **62**: 1-15.

[38]Hong Z, Hailing L, Hui M, Guijie Z, Leyan Y, Dubing Y. Effect of vitamin E supplement in diet on antioxidant ability of testis in Boer goat. *Anim Reprod Sci* 2010; **117**: 90-94.

[39]Jeong YW, Park SW, Hossein MS, Kim S, Kim JH, Lee SH, et al. Antiapoptotic and embryotrophic effects of alpha-tocopherol and *L*-ascorbic acid on porcine embryos derived from *in vitro* fertilization and somatic cell nuclear transfer. *Theriogenology* 2006; **66**: 2104-2112.

[40]Marchi S, Patergnani S, Pinton P. The endoplasmic reticulum-mitochondria connection: One touch, multiple functions. *Biochim Biophys Acta – Bioenerg* 2014; **1837**: 9461-9469.

[41]Meng J, Lv Z, Zhang Y, Wang Y, Qiao X, Sun C, et al. Precision redox: The key for antioxidant pharmacology. *Antioxid Redox Signal* 2021; **34**(14): 1069-1082.

[42]Omaye ST. Vaping-linked lung disease: Implication for pro-oxidant effect of vitamin E. *Biomed J Sci Tech Res* 2019; **23**: 17755-17556.