MJFAS MALAYSIAN JOURNAL OF FUNDAMENTAL AND APPLIED SCIENCES
PRINT ISSN: 2289-5981 | ONLINE ISSN: 2289-599X

RESEARCH ARTICLE

Palm tocotrienol-rich-fraction yields higher numbers of normal embryos whereas alpha-tocopherol produces higher preimplantation survival in murine embryos

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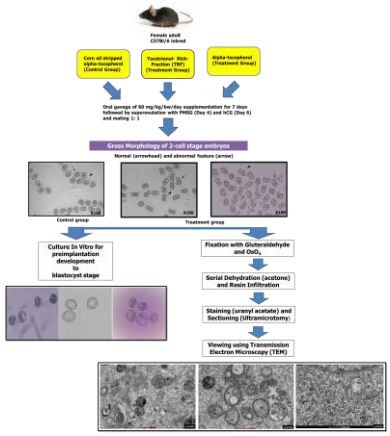
Article history
Submitted 20 March 2018
Revised 9 April 2018
Accepted 22 April 2018
Published Online 3 September 2018

Graphical Abstract



Abstract

Vitamin E contains isomers of tocotrienols and tocopherols. Studies have shown that palm tocotrienol-rich-fraction (TRF) improves preimplantation development of murine embryos. The aim of this study is to investigate the effects of TRF and α-tocopherol supplementation on preimplantation embryonic morphology, development and mitochondrial ultrastructure. Female C57Bl/6 mice were supplemented with 60 mg/kg body weight per day TRF and α-tocopherol for seven days. The females were superovulated and mated with fertile males to obtain 2-cell stage embryos. Initial assessment of normal and abnormal morphology was carried out on 2-cell embryos. The embryos were then cultured until the blastocyst stage. At the 8-cell stage, embryos were subjected to Transmission Electron Microscopy (TEM) to observe their mitochondria. Results showed that palm TRF produced significantly higher numbers of normal 2-cell embryos compared with α-tocopherol (80.9% vs 31.4%) at $p< 0.01$. Alpha-tocopherol produced higher survival rate to the blastocyst stage compared with palm TRF (42.2% vs 20.6%) at $p< 0.01$. The TRF group showed more vacuolated mitochondria at 8-cell stage compared to the α-tocopherol group, which may have contributed to a decline in preimplantation survival rates.

Keywords: Embryo development, electron transmission microscopy, mitochondria, palm Tocotrienol-Rich-Fraction (TRF), alpha-Tocopherol

INTRODUCTION

There have been numerous reports to show that Vitamin E supplementation can improve embryo quality, and therefore, developmental competence (Brigelius-Flohé et al., 2002; Guerin, 2001; Kamsani et al., 2013; Traber, 2014; Traber & Stevens, 2011). In general, assessment of embryo quality has always focused on morphology and cleavage rates. Nonetheless, other perspectives such as ultrastructural assessment of organelles such as mitochondria would be able to further validate their roles in sustaining embryo viability.

Mitochondria are important determinants of embryo developmental competence as they perform various regulatory roles during fertilization, initiation and development of preimplantation embryos (Dumollard et al., 2009). Defects in structure and number of mitochondria are likely to have adverse effects on the ability of the embryo to develop throughout the preimplantation stages. During the preimplantation period, mitochondria undergo stage-specific structural transformations in which they elongate and develop an array of cristae that transverse a matrix. Active mitochondria function to regulate oxidative phosphorylation (OXPHOS), and generates energy for the early embryos (Van Blerkom, 2009). Imbalances to the OXPHOS trigger the production of reactive oxygen species (ROS) which further induce oxidative stress and lipid peroxidation. Such condition affects cell division and metabolite transport and eventually aggravates mitochondrial damage. This may result in embryo arrest and failure of implantation.

Recent studies have shown that tocotrienols, a derivative of vitamin E is a potent lipid antioxidant (Aggarwal et al., 2010; Zingg, 2007; Zingg & Azzi, 2004). Vitamin E acts as an antioxidant and a pro-oxidant. It also functions as a signaling molecule, a regulator of gene expression, and plays a role in the prevention of cancer and other diseases (Zingg, 2015; Schneider, 2005). Since the term vitamin E encompasses a group of eight structurally related tocopherols and tocotrienols. Apart from being potent antioxidants regulating free

radicals and controlling peroxidation reactions, these isomers also display potent apoptotic action against abnormal cell growth, with no effect on normal cell function or viability (Sylvester, 2007). Several studies have reported interactions between Vitamin E isoforms at different concentrations (Yoshida, Niki, & Noguchi, 2003; Yoshida et al., 2007). For instance, outcomes from clinical trials reflect the opposing regulatory functions of α- and γ-tocopherol forms of vitamin E consumed in diets, supplements and supplement vehicles (Cool-Mills & McCary, 2010). Tocotrienols are less active but display slightly higher antioxidant activity compared to the tocopherols in membranes (Atkinson, Epand & Epand, 2008; Catalgol, Batirel & Ozer, 2011; Sen, Khanna, Roy, 2007).

The great potential of Vitamin E in reproductive studies was first discovered by Evans and Bishop (1922). Vitamin E has derivatives of 6-chromanol with an aliphatic side-chain, comprising a mixture of α-, β-, δ- and γ- tocopherols and α-, β-, δ- and γ- tocotrienols. As the phenolic hydroxyl group readily donates its hydrogen to the peroxyl radical, this feature leads to the formation of a stable lipid species. The aliphatic tail and the number of methyl species on the chromanol ring determine mobility and antioxidant capacity. The proximity of the methyl species to the hydroxyl group is also an important factor to improve antioxidant capacity. Alpha homologues of Vitamin E which possess the greatest number of methyl groups have been shown to be more effective than the other homologues. Hence, α- tocopherol has been widely used in the prevention and treatment of the diseases associated with vitamin E deficiency. However, several in vitro studies have shown that other homologues exhibit better antioxidant efficacy compared with α-tocopherol (Fujisawa & Kadoma, 2005; Stocker et al., 2003). Other non-α-tocopherols demonstrated their capability to trap electrophilic mutagens as well as to inhibit free radicals from further damaging the cells through their free aromatic ring positions (Patel et al., 2007). Hence, tocotrienols were later proposed to be equally as potent and capable as other tocopherols. Tocotrienols were further highlighted for their ability to repair nicotine-induced ultrastructural damage of the preovulatory oocyte by retaining its shape and smooth boundary of zona pellucida with tight perivitelline space (Kamsani et al., 2013; Mokhtar, Rajikin, & Zakaria, 2008).

To date, no ultrastructural studies have been carried out to examine the effects of palm TRF and α-tocopherol maternal supplementation on early preimplantation embryos. Therefore, the aim of this study was to investigate the effect of palm TRF and α-tocopherol on the morphology, development and ultrastructure of preimplantation murine embryos.

EXPERIMENTAL

The animal care, handling as well as experimentation was performed as approved by the University Committee on Animal Research and Ethics (CARE) (ACUC/CA/01/2014). Adult female C57Bl/6 mice aged 12-16 weeks were given oral gavage of 60 mg/kg/bw/day corn oil stripped of α-tocopherol (ICN Biomedicals, Aurora, Ohio, USA) as the control group, TRF (Sime Darby, M'sia, Gold Tri.ETM70) in corn oil stripped of α-tocopherol and α-Tocopherol (Sigma-Aldrich, USA) in corn oil stripped of α-tocopherol for seven days. On Day-4, they were superovulated with 5 IU Pregnant Mare Serum Gonadotropin (PMSG) (Folligon, Intervet, Holland) followed 48 hours later by 5 IU human Chorionic Gonadotropin (hCG) (Chorulon, Intervet, Holland), by intraperitoneal injection. The female mice were then mated with fertile adult male of the same strain. On Day-8, the females were euthanised, and 2-cell embryos were collected from excised oviducts into M2 medium. The normal and abnormal status of the 2-cell embryos were determined based on criteria by Khalili and Anvari (2007), where normal embryos are equal in size and number of blastomeres, with less than 10% fragmentation. Normal embryos were transferred to 50-µL droplets of M16 medium and cultured in a humidified 5% CO2 incubator at 37°C until the blastocyst stage.

For ultrastructural assessment, the 8-cell embryos were fixed in 2.5% glutaraldehyde at 4°C and rinsed with cacodylate buffer for 10 minutes each for three changes at room temperature before fixation in 1% osmium oxide for 2 hours at 4°C. This rinsing procedure was repeated. The serial dehydration in acetone was performed before they were placed in beam capsules. Agar 100 resin-acetone mixture was added to the capsules and left overnight. Finally, the mixture was replaced by 100% resin before being oven-dried at 60 °C for 48 hours. Each sample was sectioned and stained with uranyl acetate and lead citrate, before being viewed under the Transmission Electron Microscope (TEM) (Tecnai G2).

Differences between groups were determined using Chi-Square Test and were statistically significant at $P<0.001$.

RESULTS AND DISCUSSION

Images of 2-cell embryos with normal and abnormal morphology are shown in Fig. 1.

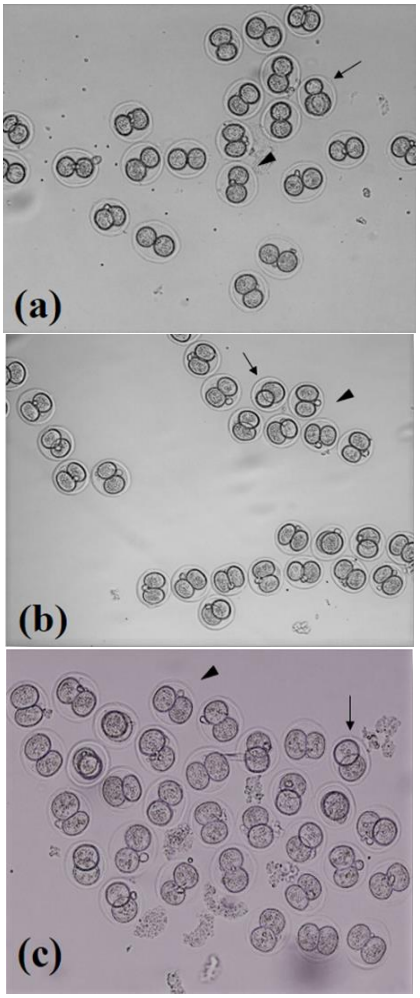


Fig. 1 Morphology of 2-cell embryos from (a) Control group; (b) TRF group; and (c) α-tocopherol group showing normal (black arrowhead) and abnormal (black arrow) embryo morphology.

The TRF group produced significantly more normal 2-cell embryos (80.9%), compared to α-tocopherol (68.6%) and control (66.7%) groups, $P < 0.01$. (Table 1).

Table 1 Morphological assessment of 2-cell embryos.

Group	No. of embryos (%)	No. of embryos (%) of normal embryos
Control (n=8)	315(100.0)	210 (66.7) [a]
TRF (n=8)	277(100.0)	224 (80.9) [b]
α-tocopherol (n=8)	264(100.0)	181 (68.6) [c]

Values in parentheses indicate percentage.
Values with different superscripts within the same column are significantly different (P <0.01)

This result implies that TRF supplementation improved initial embryo quality at the early preimplantation stage. However, during development to blastocyst stage, TRF produced the lowest viability (20.6%) when compared with control (35.2%) and α-tocopherol (42.2%) (Table 2). Tocotrienols in TRF has been reported to have antiproliferative effects on defective cells, but not on normal cells. Tocotrienol isomers have been shown to counteract cellular inflammatory response secondary to oxidative stress (Chakraborty et al., 2014).

Table 2 Development of 2-cell embryos until the blastocyst stage.

Group	No. of embryos (%)		
	Total	2-cell	Blastocyst
Control (n=8)	315 (100.0)	210 (66.7) [a]	111 (35.2) [a]
TRF (n=8)	277 (100.0)	224 (80.9) [b]	57 (20.6) [b]
α-tocopherol (n=8)	264 (100.0)	181 (68.6) [c]	112 (42.2) [c]

Values in parentheses indicate percentage.
Values with different superscripts within the same column are significantly different (P <0.01).

Further assessment was carried out on the 8-cell embryos using TEM to investigate whether this reduction rate could be associated with the changes in mitochondrial ultrastructure. Fig. 2 shows the electron micrographs of 8-cell embryos from control and treatment groups. The images indicate structural alteration, particularly of the mitochondria and endoplasmic reticulum (ER). At the 8-cell stage, immature mitochondria generally had few, peripherally localized cristae, which were observed in all groups, as reported by Sathanathan & Trounson (2000).

In Fig. 2(a) and 2(d), vacuolated mitochondria and lysosomes were observed in the control group. However, presence of densely clustered mitochondria in this group may have overcome the insufficient metabolism, resulting in higher number of embryos developing into blastocysts, when compared to TRF group (Table 2). In Fig. 2(b) and 2(e), lysosomes were observed engulfing distorted mitochondria from the TRF group. Presence of mitochondrial fission was also observed, with small segments of ER dispersing around the cytoplasm. Defects in the ultrastructure of ER could affect its synergistic association with mitochondria leading towards ions imbalance and perpetuate inflammation process (Marchi, Patergnani, & Pinton, 2014). Subsequently, such activity affects the embryo development (Van Blerkom, 2009, 2011). They may elicit mitochondrial destabilization followed by apoptosis and autophagy. Thus, mitochondria could be the indicator of the cell sensitivity towards antioxidants such as tocotrienols (Vaquero, Rickmann, & Molero, 2007).

From Fig, 2(c) and 2(f), the α-tocopherol group also displayed intra-vacuolated mitochondria surrounded by densely clustered mitochondria and long arrays of ER. This may explain the high blastocyst viability when compared with other groups. Alpha-tocopherol has been known for its potential to inhibit the production of free radical due to bioavailability of its transport proteins (Brigelius-Flohe et al., 2002; Upadhyay & Misra, 2009). It has the ability to scavenge the free radicals and to prevent formation of reactive oxygen species that could perturb the balance of cell metabolism (Müller, Theile & Böhm, 2010; Smith et al., 1999; Traber & Atkinson, 2007). Vitamin E exerts beneficial effects on the inhibition of lipid peroxidation. It is therefore useful in the prevention and treatment of various diseases where free radical-mediated oxidative stress is involved (Niki, 2014). The notion that vitamin E has very significant effects in animal models of atherosclerosis but not in humans may suggest that genes involved in the absorption, distribution and effector functions of vitamin E may be at the basis of the different responsiveness of different individuals to vitamin E supplementation (Zingg, 2015). Further investigation on this prooxidant effect by TRF requires validation through molecular analyses.

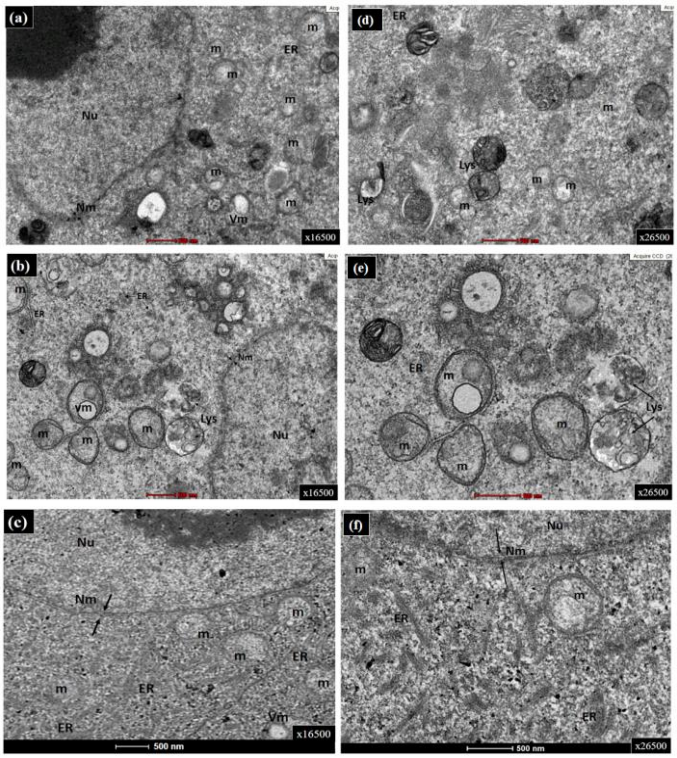


Fig. 2 Ultrastructure of 8-cell embryos classified as Control group (a & d), TRF group (b & e) and α-tocopherol group (c & f). Mitochondria (m), Vacuolated mitochondria (Vm), Nucleus (Nu), Nuclear membrane (Nm), Endoplasmic Reticulum (ER), Lysosome (Lys)

CONCLUSION

Palm tocotrienol-rich fraction supplementation produces significantly higher numbers of normal 2-cell embryos, whereas α-tocopherol contributes to higher preimplantation survival.

ACKNOWLEDGEMENT

The authors gratefully acknowledge the help of the Ministry of Higher Education, Grant No. 600-RMI/RAGS 5/3 (82/2015) and Universiti Teknologi MARA for making available its grant scheme. The authors are also thankful to the personnel of the Imaging Unit, Hospital Universiti Kebangsaan Malaysia (HUKM), Cheras, Kuala Lumpur, Malaysia for the usage of their facility.

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