Effects of Nicotine and Tocotrienol-Rich Fraction Supplementation on Cytoskeletal Structures of Murine Pre-Implantation Embryos

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Background: Cytoskeletal structures, in particular actin and tubulin, provide a fundamental framework in all cells, including embryos. The objective of this study was to evaluate the effects of nicotine, which is a source of oxidative stress, and subsequent supplementation with Tocotrienol-rich fraction (TRF) on actin and tubulin of 2- and 8-cell murine embryos.

Material/Methods: Thirty female Balb/C mice were divided into 4 groups: Group 1 received: subcutaneous (sc) injection of 0.9% NaCl; Group 2 received sc injection of 3.0 nicotine mg/kg bw/day; Group 3 received 3.0 sc injection of nicotine mg/kg bw/day +60 mg/kg bw/day TRF; and Group 4 received 60 sc injection of TRF mg/kg bw/day for 7 consecutive days. The animals were superovulated with 5 IU PMSG followed by 5 IU hCG 48 h later. Animals were cohabited with fertile males overnight and euthanized through cervical dislocation at 24 h post coitum. Embryos at the 2- and 8-cell stages were harvested, fixed, and stained to visualize actin and tubulin distributions by using CLSM.

Results: Results showed that at 2-cell stage, actin intensities were significantly reduced in the nicotine group compared to that of the control group (p<0.001). In Group 3, the intensity of actin significantly increased compared to that of the nicotine group (p<0.001). At 8-cell stage, actin intensity of the nicotine group was significantly lower than that of the control group (p<0.001). The intensities of actin in Group 3 were increased compared to that of nicotine treatment alone (p<0.001). The same trend was seen in tubulin at 2- and 8-cell stages. Interestingly, both actin and tubulin structures in the TRF-treated groups were enhanced compared to the control.

Conclusions: This study suggests that TRF prevents the deleterious effects of nicotine on the cytoskeletal structures of 2- and 8-cell stages of pre-implantation mice embryos in vitro.

MeSH Keywords: Actin Cytoskeleton • Nicotine • Tocotrienols • Tubulin

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Background

Smoking during pregnancy has been associated with a higher rate of infertility, pregnancy difficulties, and damage to the developing embryos. Studies have shown that maternal smoking during pregnancy decreases fetal growth and increases low birth weight and preterm births [1–3]. Spontaneous abortion has also been reported to be significantly increased in smokers vs. non-smokers [4].

As a main component of tobacco smoke, nicotine contributes about 90–95% of total tobacco alkaloids and it is the most important pharmacologically active compound in tobacco smoke [5]. Nicotine penetrates the placenta and enters the fetal circulation, and hence may disturb fetal development. It also causes changes in DNA methylation of genes associated with growth restriction (e.g., CYP1A1 promoter) [6–8]. Thus, it is responsible for a wide variety of negative reproductive outcomes [9,10]. In terms of oxidative stress (OS), nicotine through maternal cigarette smoking increases OS in both mother and fetus [11]. Reactive oxygen species (ROS), which are initiators of OS, can induce many cellular damages such as oxidation and fragmentation of proteins, peroxidation of membrane lipids, fragmentation of DNA, mitochondrial damage, and disruption of ion homeostasis [12,13]. Cigarette smoke contains many toxic substances and pro-oxidants that can produce ROS, for example, the SO₃⁻ anion, H₂O₂, and the OH⁻ radical, which have been reported to cause damage to the membrane structure at chromosomal [14] and ultrastructural levels [15]. Until now, reports on the effects of nicotine on the cytoskeletal structure of the embryos exposed to OS. developement, there are few reports on the role of this antioxidant on nicotine-induced embryo development. The present study investigated the effects of nicotine and the subsequent supplementation of TRF on the cytoskeletal structure (i.e., actin and tubulin).

Although a number of studies have been carried out to determine the role of tocotrienol in pre-implantation embryo development, there are few reports on the role of this antioxidant on cytoskeletal structures of the embryos exposed to OS. Immunofluorescence staining and microscopy techniques were used to examine cellular structures for actin and tubulin. A confocal laser scanning microscope (CLSM) was used to measure the intensities of cytoskeletal structures of pre-implantation embryo in mice induced with nicotine. Visual images of the cytoskeletal structure disruption that occurs with nicotine convey the dangerous effects of cigarette smoke during pregnancy, and the beneficial effect of TRF on OS suggest a potential prevention mechanism.

Material and Methods

Experimental animals and treatment

Twenty-four female mice from Balb/C strain aged 5–6 weeks and weighing 15–16 g were randomly divided into 4 groups. The palm oil TRF (Gold Tri-E 70), containing 75% tocotrienol and 25% tocopherol was purchased from Sime-Darby. All treatments were conducted between 8 am and 10 am for 7
consecutive days. The first group received 0.9% NaCl, subcutaneously (sc). The second group received 3.0 mg/kg bw/day nicotine (sc). The third group received 3 mg/kg bw/day nicotine (sc) followed by 60 mg/kg bw/day TRF oral gavage. The forth group received 60 mg/kg bw/day TRF oral gavage. All animals were kept in standard laboratory conditions at 27°C with 12-h light-dark periods and were given food pellets and water ad libitum. Ethics approval from the university Animal Care and Use Committee was obtained (ACUC 101/2015). All procedures followed the institutional animal ethics guidelines.

Origin, culture and harvest of embryos

Female mice were superovulated (intraperitoneal; ip) using pregnant mare’s serum gonadotropin (PMSG) (5 IU/kg bw) followed by human chorionic gonadotropin (hCG) (5 IU/kg bw) hormones (Intervet, Holland) 48 h post-PMSG before being mated with fertile male mice at a ratio of 1: 1. Female mice with the presence of vaginal plugs were considered to be pregnant and were selected as embryo donors. The mice were then euthanized by cervical dislocation 48 h after copulation. Fallopian tubes were excised and embryos were flushed under a dissecting microscope (Leica Zoom 2000, Japan). The 2-cell embryos were rinsed with M2 medium and cultured in vitro (37°C CO₂ incubator [5% CO₂; 95% air]) in 24-well plates (Orange Scientific, Belgium) filled with 100 µl of M16 medium (Sigma, USA) overlaid with mineral oil (Sigma, USA), until the embryos developed into the 8-cell stage. Development of embryos was observed daily under an inverted microscope (Olympus 1X81 SF-3, Japan).

Cytoskeletal staining

A total of 500 embryos at 2- and 8-cell stages were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 24 h at room temperature. The fixed cells were then incubated with 4',6-diamino-2-phenylindole dihydrochloride (DAPI) (300 nM in PBS) for 40 min to stain the nucleus before being permeabilized with 0.1% Triton X-100 in PBS for 10 min. Alexa Fluor® 635 phalloidin (6.6 µM in PBS) was added to the permeabilized cells and incubated for 1 h to stain the actin structure, and 1 µl of anti-α-tubulin conjugated antibody was diluted in 49 µl of Abdil (with ratio 1: 50) to stain the tubulin structures. The embryos were washed with PBS twice for 10 min each. Embryos were mounted on slides with Prolong® Gold Antifade reagent to minimize photo bleaching and stored at 4°C in the dark, viewed under CLSM (Leica TCS SP5). Images taken from CLSM were analyzed using LAS AF Lite software. The fluorescence intensities of the actin (excitation: 633 nm, emission: 647 nm) structures were measured according to their wavelengths.

Statistical analysis

Each experimental arm comprised 50 embryos for each stage of development. Assessment of cytoskeletal quality was based on the intensity of fluorescent probes after immunofluorescence staining and the morphology of the embryos. The percentage of embryo development at 2- and 8-cell stages indicates the survivability of the embryos. Intensities of fluorescent probes in each stage of embryo development were compared between experimental groups using the independent-samples t test. All data are expressed as mean ± SEM. In all cases, statistical analyses were performed using the Statistical Package for Social Sciences, version 20 (SPSS Inc, USA), and the differences were considered significant when p<0.05.

Results

The differences in the distribution of actin in pre-implantation embryos in all 4 groups were captured by using CLSM. Red staining signifies the actin structures of embryos stained with Alexa Fluor 635 phalloidin.

Results on the distribution of actin are summarized in Figures 1 and 2. At the 2-cell stage, actin intensities were significantly reduced in the nicotine group (12.3±0.1) (Figure 1B) compared to that of the control group (41.9±0.8) (Figure 1A), (p<0.001). Intervention with TRF (Nicotine + TRF group) (32.6±2.0) (Figure 1C) significantly reversed the intensity of actin compared to that of the nicotine group (12.3±0.1) (Figure 1B), (p<0.001). When the animals were given TRF alone (TRF group), actin intensity was higher (57.7±1.1) (Figure 1D) compared to that of nicotine + TRF group (32.6±2.0) (Figure 1C), (p<0.001).

The same trend was seen as the embryo developed further to the 8-cell stage. Actin intensity of the nicotine group (9.2±0.6) (Figure 1F) was significantly lower than that of the control group (50.1±2.5) (Figure 1E), (p<0.001). The intensities of actin in nicotine + TRF group was increased and higher (40.3±0.8) (Figure 1G) compared to that of the nicotine-treated alone group (9.2±0.6) (Figure 1F), (p<0.001). Actin intensities were significantly increased in TRF treatment (60.2±1.9) (Figure 1H) compared to that of the nicotine + TRF treatments (40.3±0.8) (Figure 1G), (p<0.001).

The tubulin structure stained with anti-α-tubulin is shown in Figures 3 and 4. At the 2-cell stage, tubulin intensities were significantly reduced in the nicotine-treated group (13.7±1.1) (Figure 3B) compared to that of the control group (55.2±1.1), (p<0.001) (Figure 3A). The intensities of tubulin were significantly reversed in the nicotine + TRF group compared to that of the nicotine group [(nicotine + TRF group vs. nicotine group; 44.3±1.2 vs. (13.7±1.1) (p<0.001) (Figure 3C, 3B)]. Results also
showed that tubulin intensities were significantly higher in the TRF treatment (86.1±1.2) (Figure 3D) compared to that of the nicotine + TRF treatment (44.3±1.2), (p<0.001) (Figure 3C). At the 8-cells stage, the cortex area became strongly stained with anti-α-tubulin and perinuclear clustering was observed in all groups, but not in the nicotine group. In the nicotine group, the tubulin intensity was significantly lower (4.2±0.7) (Figure 3F) compared to that of the control group (70.7±3.7) (Figure 3E), (p<0.001). Intervention with TRF after nicotine treatment (nicotine + TRF group) (58.4±2.5) (Figure 3G) significantly increased the intensity of tubulin compared to that of nicotine group (4.2±0.7), (p<0.001) (Figure 3F). In the group given TRF alone, the distribution of tubulin was higher (97.2±0.6) (Figure 3H) compared to that of the nicotine + TRF group (58.4±2.5) (p<0.001) (Figure 3G).

Discussion

The effects of nicotine and TRF intervention on embryonic cytoskeletal structures of actin and tubulin were studied at 2- and 8-cell stages of pre-implantation embryos. Confocal laser scanning microscope (CLSM) images showed that nicotine caused significant decrease in the fluorescent intensities of actin and tubulin at the 2- and 8-cell stages.

Actin and tubulin are very sensitive, unique and fragile structures and play major roles in normal function and development of cells [32]. Nicotine has been shown to induce oxidative stress, and exposure to nicotine may affect the cell proliferation and differentiation during embryonic development [33]. Results of the present study suggest that the reduction in the fluorescent intensity may reflect cytoskeletal damage, which may affect the embryo development later. Results from this study also proved that nicotine caused damage to the intracellular network of actin and tubulin that are essential for cytokinesis and karyokinesis. Damage to actin and tubulin may disrupt the cell cycle and alter cell viability. The exact mechanism by which nicotine causes damage to the cytoskeletal structure is unknown. However, nicotine has been proven
to elevate plasma MDA, an indicator of oxidative stress [33]. Although the present study did not measure the OS status of animals, it is possible that OS induced by nicotine causes alteration in the transmembrane protein attached to the cytoskeletal structures.

Results from this study also showed the ability of TRF to effectively reverse the deleterious effects of nicotine in 2- and 8-cell embryos, as was indicated by the higher intensities of actin and tubulin. Approximately 75% of tocotrienol in TRF is responsible for the observed benefits, and the presence of 3 trans double bonds in tocotrienol in contrast with the saturated side chain of tocopherol makes tocotrienol more readily transferred and incorporated into the cell membranes, which may aid in the repair of actin and tubulin structures. The biodistribution study of tocotrienol in null rodents showed that tocotrienol could be delivered to all organs [34]. Tocotrienol are deposited in the adipose tissues, skin, and heart, suggesting that tocotrienol is absorbed and distributed in vivo [35,36]. The additional 3 double bonds give tocotrienol greater fluidity and more mobility in the cellular membrane, which gives it specific biological and therapeutic properties compared to tocopherol [37].

The have been no similar studies on the effect of nicotine and subsequent supplementation of TRF. However, studies on actin and tubulin in cryopreserved embryos by the slow freezing method have shown that the procedure caused damage to actin and tubulin [38]. Whether the nature of the damage caused by cryopreservation is similar to that of nicotine warrants further investigation. Our results showed that supplementation with 60 mg/kg bw/day TRF is sufficient in scavenging the free radical following nicotine exposure. The present results were similar to those of a previous study done by our group, which compared 3 different doses of gamma-tocotrienol (γ-TCT) – 30 mg/kg bw/day, 60 mg/kg bw/day, and 90 mg/kg bw/day – showing that supplementation with 60 mg/kg bw/day γ-TCT was the optimal dose that maintains in vitro embryonic development in nicotine-induced oxidative stress [30]. It is interesting that the distribution of actin and tubulin was highest when mice were treated with TRF alone.

![Figure 3](image-url)  
**Figure 3.** Immunofluorescent intensities of tubulin at 2-cell (A–D) and 8-cell (E–H) stages of embryos. Control group (A, E), nicotine group (B, F), nicotine + TRF group (C, G) and TRF group (D, H) embryos stained with anti-α-tubulin for tubulin (green) were observed under a CLSM.

![Figure 4](image-url)  
**Figure 4.** Comparisons of tubulin intensity (%) between all treatment groups. Values with different superscripts are significantly different. Nic – Nicotine, TRF – tocotrienol-rich.
In the present study, we investigated 2 stages of pre-implantation embryonic development: 2- and 8-cell stages. These 2 stages reflect the variations of early stages of embryo development. The 2-cell embryos depend on their maternal factors to maintain their integrity in cellular activities. The first role of maternal factors is the processing of the male genome, which is important in embryogenesis; the second role is the elimination of maternal detritus (RNA and protein); and the third role is to activate the embryonic genome, which is important for the development of the embryo beyond 2-cell stage [39]. On the other hand, activation of the mouse embryonic genome occurs at 8-cell stage. The intensities of actin and tubulin distribution in all groups except that in the nicotine group were higher in 8-cells compared to those in 2-cells. This holds true for both actin and tubulin, and could be due to the increase in cell numbers.

Despite the finding of actin and tubulin damage following nicotine and the reversal following TRF supplementation, it is yet to be ascertained whether the numerous toxic constituents in nicotine adversely affect the cell proliferation and differentiation during embryonic development. However, a study by Syairah et al. [40] showed a significant aberration in the embryonic DNA samples following treatment with nicotine. The present findings raise questions about whether some signaling pathways or molecular mechanisms are involved behind the reported results. Therefore, future research should include comprehensive assessment of related signalling pathways and mechanisms involved.

Conclusions

Nicotine was the source of ROS that caused deleterious effects on actin and tubulin. Although measurements of OS biomarkers are not reported here, previous studies have shown that ROS originates from exogeneous nicotine and increases malondialdehyde (MDA) level, and subsequently caused damage to the cells. This study also showed that TRF may be used to improve development of nicotine-exposed embryos, and, surprisingly, suggests that TRF alone may enhance development of normal embryos. Further specific molecular data are required before TRF can be suggested as a therapeutic agent to improve embryonic cellular organization.

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References:

1. Ko TJ, Tsai LY, Chu LC et al: Parental smoking during pregnancy and its association with low birth weight, small for gestational age, and preterm birth offspring: A birth cohort study. Pediatr Neonatol, 2014; 55: 20–27
2. Harrod CS, Reynolds RM, Chasan-Taber L et al: Quantity and timing of maternal prenatal smoking on neonatal body composition: the healthy start study. J Pediatr; 2014; 165: 707–12
3. Blatt K, Moore E, Chen A et al: Association of reported trimester-specific smoking cessation with fetal growth restriction. Obstet Gynecol, 2015; 125: 1452–59
4. Lintsen A, Pasker-de Jong P, De Boer E et al: Effects of subfertility cause, smoking and body weight on the success rate of IVF. Hum Reprod, 2005; 20(7): 1867–75
5. Thielen A, Klus H, Müller L: Tobacco smoke: unraveling a controversial subject. Exp Toxicol Pathol, 2008; 60: 141–56
6. Knopik VS, Maccani MA, Francazio S, McGeary JE: The epigenetics of maternal cigarette smoking during pregnancy and effects in child development. Dev Psychopathol, 2012; 24: 1377–90
7. Lee KW, Richmond R, Hu P et al: Prenatal exposure to maternal cigarette smoking and DNA Methylation: Epigenome-wide association in a discovery sample of adolescents and replication in an independent cohort at birth through 17 years of age. Environ Health Perspect, 2015; 123: 193–99
8. Pirilä F, Guida E, Lawson F et al: Nuclear and mitochondrial DNA alterations in newborns with prenatal exposure to cigarette smoke. Int J Environ Res Public Health, 2015; 12: 1135–55
9. Schneider S, Huey C, Schütt J, Diehl K: Smoking cessation during pregnancy: A systematic literature review. Drug Alcohol Rev, 2010; 29: 81–90
10. Lisboa PC, de Oliveira E, de Moura EG: Obesity and endocrine dysfunction programmed by maternal smoking in pregnancy and lactation. Front Physiol, 2012; 3: 437–46
11. Drony A: Embryonic oxidative stress as a mechanism of teratogenesis with special emphasis on diabetic embryopathy. Reprod Toxicol, 2007; 24: 31–41
12. Bellomo G, Mirabelli, F: Oxidative stress and the cytoskeletal alterations. Ann NY Acad Sci, 1992; 663: 97–109
13. Simonian NA, Coyle JT: Oxidative stress in neurodegenerative diseases. Annu Rev Pharmacol Toxicol, 1996; 36: 83–106
14. Wang X, Falcone T, Attaran M et al: Vitamin C and Vitamin E supplementation reduce oxidative stress-induced embryo toxicity and improve the blastocysts development rate. Fert Steril, 2002; 78: 1271–77
15. Rajikin MH, Latif ES, Mar MR et al: Deleterious effects of nicotine on the ultrastructure of oocytes: Role of gamma tocotrienol. Med Sci Monit, 2009; 15(12): BR378–83
16. Barnett DK, Clayton MK, Kimura J, Bavister BD: Glucose and phosphate toxicity in hamster preimplantation embryos involves disruption of cellular organization, including distribution of active mitochondria. Mol Reprod Dev, 1997; 48: 227–37
17. Boldogh I, Vojtov N, Karmon S, Pon LA: Interaction between mitochondria and the actin cytoskeleton in budding yeast requires two integral mitochondrial outer membrane proteins, Mmm1p and Mdm12p. J Cell Biol, 1998; 141: 1371–81
18. Valderrama F, Babia T, Ayala I et al: Actin microfilaments are essential for the cytological position and morphology of the golgi complex. Eu J Cell Biol, 1996; 79: 9–17
19. Bihan TL, Pelletier D, Tancrede P et al: Effect of polar headgroup of phospholipids in their interaction with actin. J. Colloid and Interface Sci, 2005; 288: 88–96
20. Charras GT, Yarrow JC, Horton MA et al: Non-equilibration of hydrostatic pressure in blebbing cells. Nature, 2005; 435: 365–69
21. Doherty GI, Yarrow JC, Horton MA et al: Non-equilibration of hydrostatic pressure in blebbing cells. Nature, 2005; 435: 365–69
22. Ramsay JS, Rajikin MH, Chatterjee A et al: Impairment of in vitro embryonic development with a corresponding elevation of oxidative stress following nicotine treatment in mice: Effect of variation in treatment duration. Biomed Res, 2010; 21: 359–64

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23. Nesaretnam K, Meganatha, P, Veerasenan SD, Selvaduray KR: Tocotrienols and breast cancer: The evidence to date. Genes Nutr, 2012; 7: 3–9
24. Yuen KH, Wong JW, Lim AB et al: Effect of mixed-tocotrienols in hypercholesterolemic subjects. Funct Food Health Dis, 2011; 1: 106–17
25. Patel V, Rink C, Gordillo GM et al: Oral tocotrienols are transported to human tissues and delay the progression of the model for end-stage liver disease score in patients. J Nutr, 2012; 142: 513–19
26. Ahmad NS, Khalid BA, Luke DA, Ima Nirwana S: Tocotrienol offers better protection than tocopherol from free radical-induced damage of rat bone. Clin Exp Pharmacol Physiol, 2005; 32: 761–70
27. Hermizi H, Faizah O, Ima-Nirwana S et al: Beneficial effects of tocotrienol and tocopherol on bone histomorphometric parameters in sprague-dawley male rats after nicotine cessation. Calcif Tissue Int, 2009; 84: 65–74
28. Nasibah A, Rajikin MH, Nor-Ashikin MNK, Nuraliza AS: Tocotrienol improves the quality of impaired mouse embryos induced by corticosterone. In: Conf. Rec. Symposium on Humanities, Science and Engineering Research (SHUSER2012), 2012; 135–38
29. Nasibah A, Rajikin MH, Nor-Ashikin MNK, Nuraliza AS: Effects of tocotrienol supplementation on pregnancy outcome in mice subjected to maternal corticosterone administration. Journal of Oil Palm Research, 2012; 24: 1550–58
30. Kamsani YS, Rajikin MH, Nor-Ashikin MNK et al: Nicotine-induced cessation of embryonic development is reversed by γ-tocotrienol in mice. Med Sci Monit Basic Res, 2013; 19: 87–92
31. Mokhtar N, Rajikin MH, Zakaria Z: Role of tocotrienol-rich palm vitamin E on pregnancy and preimplantation embryos in nicotine treated rats. Biomed Res, 2008; 19: 181–84
32. Dobrinsky JR: Cellular approach to cryopreservation of embryos. Theriogenology, 1996; 45: 17–26
33. Feltes BC, de Faria Poloni I, Notari DL, Bonatto D: Toxicological effects of the different substances in tobacco smoke on human embryonic development by a systems chemo-biology approach. PLoS One, 2013; 8: e61743
34. Khanna S, Patel V, Rink C et al: Delivery of orally supplemented alpha-tocotrienol to vital organs of rats and tocopherol-transport protein deficient mice. Free Radic Biol Med, 2005; 39: 1310–19
35. Okabe M, Oji M, Ikeda I et al: Tocotrienol levels in various tissues of sprague-dawley rats after intragastric administration of tocotrienols. Biosci Biotechnol Biochem, 2002; 66: 1768–71
36. Ikeda S, Tohyama T, Yoshimura H et al: Dietary α-tocopherol decreases α-tocotrienol but not γ-tocotrienol concentration in rats. J Nutr, 2003; 133: 428–34
37. Suzuki YI, Tsuchiya M, Wassail SR et al: Structural and dynamic membrane properties of alpha-tocopherol and alpha-tocotrienol: Implication to the molecular mechanism of their antioxidant potency. Biochem, 1993; 32: 10692–99
38. Dasiman R, Rahman NS, Othman S et al: Cytoskeletal alterations in different developmental stages of in vivo cryopreserved preimplantation murine embryos. Med Sci Monit Basic Res, 2013; 19: 258–66
39. Li L, Zheng P, Dean J: Maternal control of early mouse development. Development, 2010; 137: 859–70
40. Syairah SMM, Rajikin MH, Sharaniza AR et al: Chromosomal status in murine preimplantation 2-cell embryos following annatto (Bixa orellana)-derived pure delta-tocotrienol supplementation in normal and nicotine-treated mice. World Appl Sci J, 2016; 34: 1855–59