TOCOTRIENOL-RICH FRACTION MODULATE THE PHOSPHOINOSITIDE 3-KINASES/AKT SIGNALING PATHWAY GENES AND PREVENT OXIDATIVE STRESS IN NICOTINE-INDUCED PRE-IMPLANTATION EMBRYOS

NURUL HAMIRAH KAMSANI1,2, SHARANIZA AB-RAHIM1,3, YUHANIZA SHAFINIE KAMSANI1,2,4, NOR ASHIKIN MOHAMED NOOR KHAN1,2,4, MOHD HAMIM RAJIKIN1,2,4

1Department of Physiology Faculty of Medicine, Institute of Medical Molecular Biotechnology, Universiti Teknologi MARA, Cawangan Selangor, Kampus Sungai Buloh, 47000 Sungai Buloh, Selangor, Malaysia. 2Department of Physiology, Faculty of Medicine, Universiti Teknologi MARA, Cawangan Selangor, Kampus Sungai Buloh, 47000 Sungai Buloh, Selangor, Malaysia. 3Department of Biochemistry and Molecular Medicine, Faculty of Medicine, Universiti Teknologi MARA, Cawangan Selangor, Kampus Sungai Buloh, 47000 Sungai Buloh, Selangor, Malaysia. 4Department of Physiology, Faculty of Medicine, Materinofetal and Embryo Research Group, Universiti Teknologi MARA, Cawangan Selangor, Kampus Sungai Buloh, 47000 Sungai Buloh, Selangor, Malaysia. Email: hamim400@salam.uitm.edu.my

Received: 24 January 2019, Revised and Accepted: 25 July 2019

ABSTRACT

Objective: This study aimed to determine the effects of the tocotrienol-rich fraction (TRF) on the regulations of phosphoinositide 3-kinases (PI3K)/Akt pathways related genes in preimplantation embryos induced by nicotine (Nic).

Methods: Twenty-four female BALB/c mice were divided into four groups with Nic and TRF supplementation for 7 consecutive days. Animals were superovulated before mating with fertile males. Plasma malondialdehyde, superoxide dismutase, catalase, and glutathione peroxidase were determined and analyzed accordingly. Embryos with two and eight blastomeres were assessed for gene expression analysis.

Results: The levels of endogenous antioxidative enzymes for the group with TRF intervention and TRF-only group showed no significant changes when compared to the control group. The level of oxidative stress (OS) biomarkers was also significantly decreased when compared to the Nic-induced group. At 2-cell stage, Pten gene was significantly upregulated while Akt1, GSK3β, and Mapk1 were significantly downregulated almost similar to the baseline (control) in the Nic-induced mice. Intervention with TRF resulted in a significant downregulated of Pten gene followed by a significant upregulation of other genes. The same pattern was shown at the 8-cell stage.

Conclusion: This showed that TRF evidently has OS protection capacity and it could be through modulating the PI3K/Akt signaling pathway.

Keywords: Tocotrienol-rich fraction, Nicotine, Preimplantation embryo.

INTRODUCTION

Tocotrienol-rich fraction (TRF), an unsaturated form of Vitamin E, is an important supplement in nutrition. Research emerging on tocotrienol began in 1991 when the biology of tocotrienol was extensively studied. It has been proven that these unique isoforms of Vitamin E have a wide range of physiological properties which include as an antioxidant [1], antihypertensive [2], anticancer agent [3-6], and capable to reduce cardiovascular disease risk by lowering total cholesterol and low-density lipoprotein [7]. It also has neurodegenerative and neuroprotective effects [8], as well as promote bone formation and repair in damaged bone caused by long-term smoking [9,10]. A study by Mokhtar et al. (2009), on the animal model showed that oral supplementation with tocotrienol increased success rate of hatched blastocyst formation [11].

Our previous studies have reported that tocotrienol was able to reverse the oxidative stress (OS) effects on pre-implantation mouse embryos induced by corticosterone [12,13] and fetal development and survivability [14].

The development of preimplantation or cleavage-stage embryos begins when the nucleus starts to duplicate itself and divide into cells as blastomeres. This process is known as mitosis, which happens in between stage of fertilization and blastocysts formation. They are also normally referred to as 2-cells, 4-cells, 8-cells, morulae, compacted morulae, blastocysts, and hatched blastocysts based on their stage of the development [15,16]. Normal embryo's characteristics consist of the existence of zona pellucida, compact and even ooplasm, even blastomere cells or presence of apparent and unifrom blastomere membranes. Abnormal embryo's characteristics include only one blastomere (undivided or not yet divided), lack of zona pellucida, empty zona pellucida or ununiformed size of blastomeres [17].

OS has become an area of great concern for clinicians and scientists because this pathway of programmed deterioration has also resulted in poor fertilization, poor embryonic development, pregnancy loss, and birth defects. Free radicals, such as reactive oxygen species (ROS), are generated as by-products of cellular metabolism. Common forms of ROS include hydrogen peroxide (H$_2$O$_2$), superoxide (O$_2^- $), and nitric oxide (NO). They carry out important cellular functions under normal physiological conditions such as in cell's signaling/feedback, proliferation, immunity or inflammation, oxygen sensing, and differentiation. ROS is capable of inflicting significant damage to cell structures resulting in a pathological condition in these cells. As they can be highly toxic molecules, their levels are regulated by the endogenously produced ROS-scavenging enzymes such as glutathione peroxidase (GPX), catalase (CAT), and superoxide dismutase (SOD) [18,19] or exogenously derived micronutrients such as polyphenols, Vitamin C, carotenoids, and Vitamin E including TRF [20,21]. OS arises following an imbalance between pro-oxidants and antioxidants, which impair intracellular homeostasis. OS causes damage to the cytoskeletal ultrastructure in normal cells [22,23], pre- and post-implantation embryos, in which their subsequent
development could be at stake [24,25]. Cigarette smoke, with its major content, consist of the alkaloid nicotine (Nic), contains pro-oxidants that may raise ROS. This has been reported to damage the membrane structure at the chromosomal [26] and ultrastructural levels [27].

Nic addiction during pregnancy has been reported to decrease offspring’s birth weight leading to infant death in the world [26,28]. Effects of Nic from cigarette smoke are not only limited to active smokers but also are evident in passive smokers too. At the cellular level, Nic increases intracellular free calcium in the endoplasmic reticulum (ER); an important ultrastructure involved in protein and lipid synthesis of a cell. Therefore, it has been consistently proven that Nic increases ER stress response leading to embryonic cell death [27,29,30]. Nic also alters the ultrastructure of oocytes [27] and possibly trigger related inflammatory pathways [31,32].

Abnormal embryo development resulted from a disturbance in the mechanism of DNA modulation. This cellular disorganization is caused by dysfunctionality in the normal genes and kinases which are responsible for cell proliferation. Normally, following DNA damage detection, repair pathways are activated through transient cell cycle arrest in the G1/S phase. If this unable to be mitigated, cell cycle arrest will either be permanent, or apoptosis is triggered to eliminate the damaged cells. However, if neither of them occurred, then this will lead to a condition of uncontrolled cell growth.

Phosphoinositide 3-kinases (PI3Ks) are a family of signaling enzymes which regulate a variety of important cellular functions, including cell growth, cell cycle progression, apoptosis, migration, metabolism, and vesicular trafficking [33,34]. The realization that PI3K signaling is disrupted at multiple levels has prompted researchers to develop targeted therapies against individual enzymes involved in this signaling cascade [35,36]. Some of the proposed mechanisms for the antiapoptotic effect of activated Akt include the inhibition of proapoptotic Bcl-2 family proteins, downregulation of death receptors, and enhancement of the glycolytic rate [37]. They mediate the effects on cellular responses, including apoptosis, growth, and cell cycle regulation [38,39]. Using Nic-induced preimplantation mice embryos, this study is carried out to investigate further on the mechanism of TRF and its role in maintaining DNA integrity through PI3K/Akt signaling pathway.

METHODS

Experimental animals and treatment

Twenty-four (24) female mice from BALB/C strain aged 5–6 weeks weighing 15–16 g were randomly divided into four groups. Palm oil TRF (Gold TriE 70), containing 75% TRF, and 25% tumor control probabilities (Sime Darby) were used. All treatments were conducted for 7 consecutive days. Group 1 (G1) received 0.9% NaCl subcutaneously (sc). Group 2 (G2) received 3.0 μl 2×CellsDirect master mix, 1 μl Exonuclease I master mix and 10 reverse primers mixed at a final concentration of 500 nM and 1 μl Superase-In (Ambion, USA). Each reaction mix contained 1.25 μl primers mix (10 forward primers and 1 reverse primer) and 10 μl of preamplified single cell cDNA. The reactions were initiated by adding 20 μl of diluted hydrogen peroxide to each sample. Standard curve for β-actin was prepared by diluting 10 μl of β-Actin Formaldehyde standard with 9.99 ml of diluted sample buffer to make 4.25 mM Formaldehyde stock solution. A series of standard solution ranging from 5 to 75 μM. The value was expressed as nmol/min/g.

SOD activity was measured by subjecting 10 μl of sample to 200 μl of the diluted radical detector. The reactions were initiated by adding 20 μl of diluted xanthine oxidase to all samples. A standard curve for the reaction was prepared by diluting 20 μl of SOD standard with 1.98 ml of diluted sample buffer. A series of standard solution ranging from 0.005 to 0.05 (U/ml). The absorbance was read at 440–460 nm. The value was expressed as U/g.

Preparation of preamplified single cell cDNA

Collected embryos were cultured in a culture dish filled with 100 μl of M16 media (Sigma, USA) and overlaid with mineral oil (Sigma, USA) to further develop the embryos until 2- and 8-cell stages. Cultures were maintained in a CO2 incubator at 37°C (5% CO2, 95% air) (Memmert, Germany). The daily observation was done using an inverted microscope (Olympus 1×81 SF-3, Japan) until the embryos reach the desired stages; 2- and 8-cell. Single blastomeres were isolated by first removing the zona pellucida with proteinase (Sigma, USA), followed by separating the cells with Trypsin (Sigma, USA). Single cells were picked with the help of a mouth pipette and a finely pulled with a glass capillary.

Specific target gene amplification was performed by pipetting the single cells of each sample directly into 0.1 ml nuclease-free polymerase chain reaction (PCR) tubes containing the CellsDirect™ One-step quantitative reverse transcription-PCR Kit reaction mix (Invitrogen, USA). Each reaction mix contained 1.25 μl primers mix (10 forward and 10 reverse primers mixed at a final concentration of 500 nM each) of the target genes (Table 1). 2.5 μl 2×CellsDirect master mix 0.1 μl CellsDirect Enzyme mix, 0.1 μl Supersense-In (Ambion, USA), and 0.55 μl DNA Reasensation Buffer (TEKnova, USA) amounted to 4.5 μl total volume. Reverse transcription was performed by incubation in a standard thermal cycler (Bio-Rad, USA) for 15 min at 50°C, followed by 2 min at 95°C and subsequently 18 cycles of PCR amplification (15 s at 95°C and 4 min at 60°C). Residual primers were removed by adding 2 μl Exonuclease I master mix to the pre-amplified reactions. The Exonuclease I Master mix consisted of 0.4 μl Exonuclease I (New England

| Target genes | Forward primers | Reverse primers | Design Ref Seq. |
|--------------|-----------------|-----------------|----------------|
| Akt1         | GCGGTTGACATCTCATGAGCA | GAGACTCTAGGACTCTGCTG | NM_009652.N |
| Pten         | AACGGTCTCGTCCTGCTCGGTTTAA | GAGACTATTAGACAGAGCCAAA | NM_008960.2 |
| Mapk1        | TGGCTGGACAGAGACAGAAATA | GCGACTTTCACTCGTGGTGA | NM_00103863.1 |
| Gsk3β        | GCAATGTTGACACTACTGGTGGTAAAA | GCGACTTTCACTCGTGGTGA | NM_019827.N |

Table 1: List of the targeted genes and their forward and reverse primers.
BioLabs, USA). 0.2 μl 10× reaction buffer (provided with enzyme), and 1.4 μl nuclease-free water. The samples were incubated for 30 min at 37°C, followed by 15 min at 80°C. The pre-amplified reaction products were diluted five-fold and stored at -20°C until further use.

Single-cell quantitative PCR (qPCR) with BioMark 192.24 GE dynamic arrays
Sample Pre-Mix solutions were prepared in two 96-well plates for the 192 samples, using 2.7 μl of the preamplified cDNA, mixed with 3.0 μl 2×Sso Fast EvaGreen Supermix with Low ROX (Bio-Rad Laboratories, USA) and with 0.3 μl 20×DNA binding dye sample loading reagent (Fluidigm, USA). The assay mix for the 10 assays was prepared by mixing 3.0 μl 2×assay loading reagent (Fluidigm, USA) with 2.7 μl 1×DNA suspension buffer (Teknova, USA) and 0.3 μl 100 μM each of forward and reverse primer mix. A 192.24 GE dynamic array chips were loaded and run on a BioMark system as described by the manufacturer (Fluidigm, USA).

Data processing and analysis
Data from the BioMark qPCR system were processed with real-time PCR Analysis Software (Fluidigm, USA), including quality control of the experiment and identification of unspecific products based on the product melting temperature. The Ct values obtained from the BioMark™ System were converted into relative expression levels by subtracting the values from the assumed baseline value of 30 (inverted Ct values). The resulting values (10 genes and 192 samples) were normalized to the average signal for the endogenous reference genes Hprt, Actb, and Gapdh, by subtracting the average inverted Ct value for the three reference genes from the respective expression value.

Statistical analysis
Data were expressed as the mean±standard error of the mean. Statistical analyses were performed using the Statistical Package for the Social Sciences, version 23 (SPSS Inc, USA). The differences were considered significant when p<0.05.

RESULTS
TRF reduced the OS level
OS level of MDA, GPx, CAT, and SOD is shown in Fig. 1a-d. Plasma MDA was increased in 3.0 mg/kg bw/day Nic treated group (103.4±30.01 nmol/l vs. 224.1±21.12 nmol/l; p<0.05) with corresponding decreases in plasma GPx (41.17±3.23 nmol/min/ml vs. 165.39±30.02 nmol/min/ml; p<0.05), CAT (20.64±1.37 nmol/min/ml vs. 11.16±1.98 nmol/min/ml; p<0.05), and SOD (247.5±22.25 U/ml vs. 101.31±19.38 U/ml; p<0.05), compared to controls. Concurrent treatment of Nic and TRF has normalized plasma MDA (111.7±29.11 nmol/l vs. 104.11±27.04 nmol/l), GPx (434.63±91.2 nmol/min/ml vs. 400.8±95.14 nmol/min/ml), CAT (23.75±2.15 nmol/min/ml vs. 20.97±2.02 nmol/min/ml) and SOD (272.2±29.91 U/ml vs. 190.97±34.16 U/ml) close to controls.

Gene expression in 2-cell embryos
Data on the fold change values of the studied genes in 2-cell embryos are shown in Fig. 2. Results of G2 following treatment with 3.0 mg/kg bw/day of Nic showed significant upregulated Pten gene at 0.26-fold. However, Akt1, Gsk3β, and Mapk1 genes were significantly downregulated at 0.57-fold, 0.33-fold, and 0.51-fold, respectively. Intervention with TRF in G3 and G4 both resulted in a significant downregulated Pten gene with 0.7-fold in G3 and 0.4-fold in G4. This was further followed by a significant upregulation at 1.55-fold (G3) and 1.72-fold (G4) of Akt1 gene. Concurrent supplementation of Nic with TRF in G3 also resulted in upregulations of Gsk3β and Mapk1 genes at 0.1-fold and 0.32-fold, respectively. Data in G4 showed the significant upregulation (p<0.001) of Gsk3β at 2.51-fold and Mapk1 at 3.1-fold.

Gene expression in 8-cell embryos
Data on the fold change values of the studied genes in 8-cell embryos in Fig. 3. Embryos that develop into 8-cell stage in G2 following treatment with 3.0 mg/kg bw/day of Nic showed the significant upregulated Pten gene at 0.38-fold; however, significantly downregulated for Akt1, Gsk3β, and Mapk1 genes at 0.37-fold, 0.26-fold, and 0.07-fold, respectively (p<0.001). Intervention with TRF in G3 resulted in the significant downregulation of Pten gene at 0.26-fold (p<0.05). However, the expression showed upregulated for the Akt1, Gsk3β, and Mapk1 genes at 0.1-fold, 0.03-fold, and 0.04-fold, respectively. Supplementation with TRF alone in G4 resulted in the significant downregulation of Pten gene expression at 0.17-fold, whereas the other genes were upregulated.

DISCUSSION
Nic as an OS inducer has been well established in the previous studies [14,27,40,41]. This study has further confirmed the effects of Nic in developing embryos, and showed that TRF was able to counteract the effects of Nic.

Fig. 1: (a) Plasma malondialdehyde; (b) catalase; (c) glutathione peroxidase; and (d) superoxide dismutase in animals across all groups. (* ) indicates significant value at p<0.05 when compared to control.

4th International Conference on Pharmacy and Pharmaceutical Science (ICPPS) 2019
Nic is an OS inducer, as shown in our data on the increase of OS markers level in the Nic group. Nic is well-known to contain massive amounts of oxidants on its own, and infiltrated neutrophils and macrophages are another major sources of ROS responsible for lipid peroxidation (LP) [42]. This study has further shown a reduction of plasma LP accompanied by restoration of enzymatic antioxidants in TRF supplemented group showing the potency of TRF in combating Nic-induced OS. The ability of TRF to effectively prevent the effects of Nic in 2- and 8-cell embryos was indicated by the high intensities of actin and tubulin as previously reported by our group [41]. Approximately 75% of TRF is responsible for the observed benefits, and the presence of three trans double bonds in TRF makes it more readily transferred and incorporated into the cell membranes, which may aid in the repair of actin and tubulin structures.

This study evidently highlighted the protective effects of TRF in reducing the ROS-induced OS by the marked decrease in the level of MDA in vivo. This has been supported earlier by the bio-distribution study of TRF in null mouse showed that TRF can be delivered to all organs [8] including adipose tissues, skin, and heart, further [8,43] suggesting that TRF is well absorbed and distributed in vivo. This is due to the additional three double bonds in TRF which give the greater fluidity and more mobility in the cellular membrane and further gives specific biological and therapeutic properties [8,44]. Our finding matched to those observed in an earlier study which has reported higher concentrations of SOD, CAT, and GPx but lower levels of plasma MDA following TRF treatment on exercise endurance and OS in forced swimming rats [45].

The present study was also conducted to determine the effects of TRF supplementation on the PI3K/Akt genes regulation in Nic-induced mice pre-implantation embryos. Our result shows that Pten gene was significantly upregulated while the other genes (Akt1, Gsk3β, and Mapk1) which associated with PI3K/Akt signaling were significantly downregulated in the Nic-induced groups at 2-cell stage compared to the control group. This data showed the detrimental effects on Nic whereby it these genes which involve in PI3K/Akt pathway may affect the continuity of the cell and nucleus division and may eventually disrupt the development of the pre-implantation embryos. We have also documented previously that the actin and tubulin arrangement was also disrupted by Nic in the pre-implantation embryos, which in turn, affecting the embryonic development [41]. Disruption of the cytoskeletal arrangement could be due to the modulation of the PI3K/Akt genes, as shown in this study.

In contrast to the Nic-induced group, TRF supplementation in the Nic-induced group, Pten gene was significantly downregulated while the other genes were significantly upregulated suggesting the TRF potency in preventing the negative effect of Nic in the embryo’s development at the 2-cell stage. This has further confirmed earlier in this study whereby the supplementation of TRF in the Nic-induced group significantly reduced the OS enzymes almost the same as the control groups. The same pattern was seen in the gene expression at the 8-cell stage. These data suggest the positive role of TRF in modulating the expression of the genes in preventing the Nic-induced OS. Pten has crucial roles in cell proliferation, survival, differentiation, and migration. Expression of this gene was significantly upregulated in Nic group suggesting that Pten gene might influence the attenuation of the embryonic growth from as early as at 2-cell stage. Control and TRF groups showed an increase in Akt1, Gsk3β, and Mapk1 genes. It is suggested that the increase in these genes may trigger the activation of PI3K/Akt pathway, leading to an increase in the proliferation rate throughout the developmental stages. TRF prevents upregulation of Pten that was induced by Nic. This explains the reduced number of retrieved embryos and decreased number of hatched blastocysts in mice treated with Nic.

The present study focuses on two stages of preimplantation embryonic development, which are 2- and 8-cell stages. These two stages show the differences between early stages of embryo development. At the 2-cell stage of development, embryos depend on their maternal genome factors to maintain their integrity in cellular functions. The first role of maternal factors is the processing of the male genome, which is significant in embryogenesis; the second role is the removal of maternal detritus (RNA and protein); and the third role is to initiate the embryonic genome, which is important for the development of the embryo beyond the 2-cell stage [46]. Meanwhile, activation of the mouse embryonic genome happens at the 8-cell stage.

Further studies are warranted to gain more information and understandings on the other possible physiological changes and molecular mechanisms that may involve in coregulate the protective effects of TRF against embryonic DNA damage.

CONCLUSION
TRF was shown to modulate the PI3K/Akt pathway associated genes in the 2- and 8-cell preimplantation embryos. The effect of TRF in regulating the genes expression back to the baseline in Nic-induced group could indicate the OS prevention properties of TRF mechanism is through the PI3K/Akt pathway.

ACKNOWLEDGMENT
This research is supported by the Ministry of Education, Malaysia (600-RMI/RAGS 5/3 [44/2014]), (600-RMI/FRGS 5/3 [0015/2016]) and
REFERENCES

1. Serbinova E, Kagan V, Han D, Packer L. Free radical recycling and intracellular mobility in the antioxidant properties of alpha-tocopherol and alpha-tocotrienol. Free Radic Biol Med 1991;10:263-75.

2. Koba K, Abe K, Ikeda I, Sugamo M. Effects of alpha-tocopherol and tocotrienols on blood pressure and holoionic acid metabolism in the spontaneously hypertensive rat (SHR). Biosci Biotechnol Biochem 1992;56:1420-3.

3. Rahmat A, Ngah WZ,莎玛娜 NA, Gapor A, Abdul Kadir K. Long-term administration of tocotrienols and tumor-marker enzyme activities during hepatocarcinogenesis in rats. Nutrition 1993;9:229-32.

4. Goh SH, Hiew NF, Norhanom AW, Yadav M. Inhibition of tumour promotion by various palm-oil tocotrienols. Int J Cancer 1994;57:529-31.

5. Nesaretnam K, Guthrie N, Chambers AF, Carroll KK. Effect of vitamin E on human breast cancer cell line in culture. Lipids 1995;30:1139-43.

6. Nesaretnam K, Meganathan P, Veerasenan SD, Selvaduray KR. Vitamin E supplementation on pregnancy outcome in mice subjected to maternal corticosterone administration. J Oil Palm Res 2012;24:1550-8.

7. Chatterjee A. Nicotine-induced cessation of embryonic development in mice treated rats. Biomed Res 2008;19:181-4.

8. Patel V, Rink C, Gordillo GM, Khanna S, Gnyawali U, Roy S, 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;142:513-9.

9. Ahmad NS, Khalid BA, Luke DA, Ima Nirwana S. Tocotrienol offers protective effects of on human breast cancer cell line. Curr Med Chem 2005;12:761-70.

10. Hermzi H, Faizah O, Ima-Nirwana S, Ahmad Nazrun S, Norazlina M. Beneficial effects of tocotrienol on tocopherol and tocotrienol on bone histomorphometric parameters in sprague-dawley male rats after nicotine cessation. Curr Med Chem 2009;16:65-74.

11. 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;19:181-4.

12. Nasibah A, Rajikin MH, Nor-Ashikin MN, Nuraliza AS. Tocotrienol Improves the Quality of Impaired Mouse Embryos Induced by Corticosterone. Kota Kinabalu: CHUSER 2012-2012 IEEE Colloquium on Humanities, Science and Engineering Research; 2012. p. 135-8.

13. Nasibah A, Rajikin MH, Nor-Ashikin MN, Nuraliza AS. Effects of tocotrienol supplementation on pregnancy outcome in mice subjected to maternal corticosterone administration. J Oil Palm Res 2012;24:1550-8.

14. Kamsani YS, Rajikin MH, Mohamed Nor Khan NA, Abdul Satar N, Chatterjea A. Nicotine-induced cessation of embryonic development is reversed by γ-tocotrienol in mice. Med Sci Monit Basic Res 2013;19:87-92.

15. Snyder JM. Department of Anatomy and Cell Biology, University of Iowa. Lecture Medical Cell Biology. Iowa: Fertilization and Implantation; 2001.

16. Benkhalfila M, Menezo Y. Geneva Foundation for Medical Research and Education. Gamete and Embryo Quality, Role in Fertilization Failures and Reproductive Pathology: The Contribution of Fluorescent in Sito Hybridization (FISH). Geneva: GFMER; 2003.

17. Ertezid G, Storeng R. Adverse effects of gonadotropin treatment on pre- and postimplantation development in mice. J Reprod Fertil 1992;96:649-55.

18. Hemachandra LM, Chandrasekaran A, Melendez JA, Hempel N. Regulation of the Cellular Redox Environment by Superoxide Dismutases, Catalase, and Glutathione Peroxidases During Tumor Metastasis. Redox ActiVe Therapeutics. Switzerland: Springer; 2016. p. 51-79.

19. Lei XG, Zhu JH, Cheng WH, Bao Y, Ho YS, Reddi AR, et al. Paradoxical roles of antioxidant enzymes: Basic mechanisms and health implications. Physiol Rev 2016;96:307-64.

20. Grimm MO, Regner L, Mett J, Stahlmann CP, Schorr P, Nelke C, et al. Tocotrienol affects oxidative stress, cholesterol homeostasis and the amyloidogenic pathway in neuroblastoma cells: Consequences for Alzheimer’s disease. Int J Mol Sci 2016;17:18709.

21. Tan JK, Then SM, Mazlan M, Jamal R, Ngah WZ. Vitamin E, γ-tocotrienol, protects against buthionine sulfoximine-induced cell death by scavenging free radicals in SH-SY5Y neuroblastoma cells. Nutr Cancer 2016;68:507-17.

22. Yao Y, Lacroix D, Mok AF. Effects of oxidative stress-induced changes in the actin cytoskeletal structure on myoblast differentiation under compressive stress: Confocal-based cell-specific finite element analysis. Biomach Mechanobioch 2016;15:1495-508.

23. Huang X, Chen L, Liu W, Qiao W, Wu K, Wen J, et al. Involvement of oxidative stress and cytoskeletal disruption in microtubin-induced apoptosis in Ck1 cells. Anticu Toxicol 2015;165:41-50.

24. Zhang Y, Qian D, Li Z, Huang Y, Wu Q, Ru G, et al. Oxidative stress-induced DNA damage of mouse zygotes triggers G2/M checkpoint and phosphorylates cdc25 and cdc2. Cell Stress Chaperones 2016;21:687-96.

25. Jadhav JT, Kengar S. Influence of hydrogen peroxide induced oxidative stress on survival rate of early chick embryo development. Int J Bioassays 2016;5:4603-6.

26. Niu Z, Xie C, Wen X, Tian J, Yan S, Jia D, et al. Potential pathways by which maternal second-hand smoke exposure during pregnancy causes full-term low birth weight. Sci Rep 2016;6:24987.

27. Rajikin MH, Latif ES, Mar MR, Top AG, Mokhtar NM. Deleterious effects of nicotine on the ultrastructure of oocytes: Role of gamma-tocotrienol. Med Sci Monit Basic Res 2013;19:BR378-80.

28. Knopik VS, Maccioni MA, Francozio S, McGeorge JE. The epigenetics of maternal cigarette smoking during pregnancy and effects on child development. Dev Psychopathol 2012;24:1377-90.

29. Gu YH, Li Y, Huang XF, Zheng JF, Yang J, Mao H, et al. Reproductive effects of two neonicotinoid insecticides on mouse sperm function and early embryonic development in vitro. PLoS One 2013;8:e70112.

30. Hirata N, Yamada S, Asanagi M, Sekino Y, Kanda Y. Nicotine induces mitochondrial fission through mitofusin degradation in human multipotent embryonic carcinoma cells. Biochem Biophys Res Commun 2016;470:500-7.

31. Bellomo G, Mirabelli F. Oxidative stress and cytoskeletal alterations. Ann N Y Acad Sci 1992;663:97-109.

32. Wang X, Falcone T, Attaran M, Goldberg JM, Agarwal A, Sharma RK, et al. Vitamin C and Vitamin E supplementation induce oxidative stress-induced embryo toxicity and improve the blastocyst development rate. Fertil Steril 2002;78:1272-7.

33. Engelmann JA, Luo J, Cantley LC. The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. Nat Rev Genet 2006;7:60-9.

34. Garcia Z, Kumar A, Marques M, Cortes I, Carrera AC. Phosphoinositide 3-kinase controls early and late events in mammalian cell division. EMBO J 2006;25:655-61.

35. Shaw RJ, Cantley LC. PI3 kinases in cancer: From oncogene artifact to leading cancer target. Sci STKE 2006;2006:pe52.

36. Zhao JJ, Roberts TM. PI3 kinases in cancer: From oncogene artifact to leading cancer target. Sci STKE 2006;2006:pe52.

37. Lacroix D, Mak AF. Effects of oxidative stress-induced changes in the actin cytoskeletal structure on myoblast differentiation under compressive stress: Confocal-based cell-specific finite element analysis. Biomach Mechanobioch 2016;15:1495-508.
42. Kirkham PA, Barnes PJ. Oxidative stress in COPD. Chest 2013;144:266-73.
43. Uchida T, Abe C, Nomura S, Ichikawa T, Ikeda S. Tissue distribution of α- and γ-tocotrienol and γ-tocopherol in rats and interference with their accumulation by α-tocopherol. Lipids 2012;47:129-39.
44. Atkinson J, Epand RF, Epand RM. Tocopherols and tocotrienols in membranes: A critical review. Free Radic Biol Med 2008;44:739-64.
45. Lee SP, Mar GY, Ng LT. Effects of tocotrienol-rich fraction on exercise endurance capacity and oxidative stress in forced swimming rats. Eur J Appl Physiol 2009;107:587-95.
46. Li L, Zheng P, Dean J. Maternal control of early mouse development. Development 2010;137:859-70.