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Research Article

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

NURUL HAMIRAH KAMSANI[1,2], SHARANIZA AB-RAHIM[1,3], YUHANIZA SHAFINIE KAMSANI[1,2,4], NOR ASHIKIN MOHAMED NOOR KHAN[1,2,4], MOHD HAMIM RAJIKIN[1,2,4]*

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

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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.

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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 uniform 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_2O_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 the DNA integrity through PI3K/Akt signaling pathway.

METHODS

Experimental animals and treatment

Twenty-four (24) female mice from BALB/C strain aged 5–6 w weighing 15–16 g were randomly divided into four groups. Palm oil TRF (Gold Tri.E 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 mg/kg body weight (bw)/day Nic (sc). Group 3 (G3) received 3.0 mg/kg bw/day Nic (sc) followed by 60 mg/kg bw/day TRF oral gavage (OG). Group 4 (G4) received 60 mg/kg bw/day TRF (OG). Ethics approval from UiTM Animal Care and Use Committee were obtained (ACUC 101/2015). All procedures adhered to Institutional Animal Ethics guidelines.

Origin, culture, and harvest of embryos

All the female mice were superovulated (intraperitoneal) using 5 IU/kg bw pregnant mare serum gonadotropin (PMSG) followed by 5 IU/kg bw human chorionic gonadotropin hormones (Intervet, Holland) 48 h post-PMSG. Animals were then mated with fertile male mice. Only animals with the presence of vaginal plugs were proceed with this study. The mice were euthanized by cervical dislocation 48 h after copulation. Plasma was collected to determine the OS biomarkers and embryos at 2-and 8-cell stages were collected for gene expression analysis.

Determination of OS biomarkers

OS biomarkers were determined using respective commercial kit assays (Cayman Chemical, USA). Malondialdehyde (MDA) level was measured by subjecting 500 µl of plasma to 500 µl extract R saturated methanol and 1 ml of cold chloroform. Standard was prepared by aliquot the lipid hydroperoxide standard to the chloroform-methanol mixture. The absorbance was read at 500 nm. The value was expressed as nmol/l.

GPx activity was measured by subjecting 20 µl of plasma or standard to 100 µl of Assay Buffer and 50 µl of cosubstrate mixture. The reaction was initiated by adding 20 µl of cumene hydroperoxide. The absorbance was read at 340 nm. The value was expressed as nmol/min/g.

CAT activity was measured at 540 nm by adding 20 µl of sample to 100 µl of diluted assay buffer and 30 µl of methanol. The reactions were initiated by adding 20 µl of diluted hydrogen peroxide to each sample. Standard curve for CAT activity was prepared by diluting 10 µl of CAT 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 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 CO_2 incubator at 37°C (5% CO_2; 95% air) (Memmert, Germany). The daily observation was done using an inverted microscope (Olympus 1×81 SF-3, Japan) until the embryos reach the desire 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 Superase-In (Ambion, USA), and 0.55 µl DNA Resuspension 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

Table 1: List of the targeted genes and their forward and reverse primers

Target genes	Forward primers	Reverse primers	Design Ref Seq.
Akt1	CGTTGGCATACTCCATGACA	AGAACTCTAGGCATCCCTTCC	NM_009652.N
Pten	AAGTTCTAGCTGTGGTGGGTTA	GAGACATTATGACACCGCCAAA	NM_008960.2
Mapk1	TGCAGCCCACAGACCAAATA	CGTTGGTACAGAGCTCCAGAA	NM_001038663.1
*Gsk3*β	GGAGTTGCCACTACTGTGGTTA	GCAGCCTTCAGCTTTTGGTA	NM_019827.N

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 (411.71±63.23 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 of *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

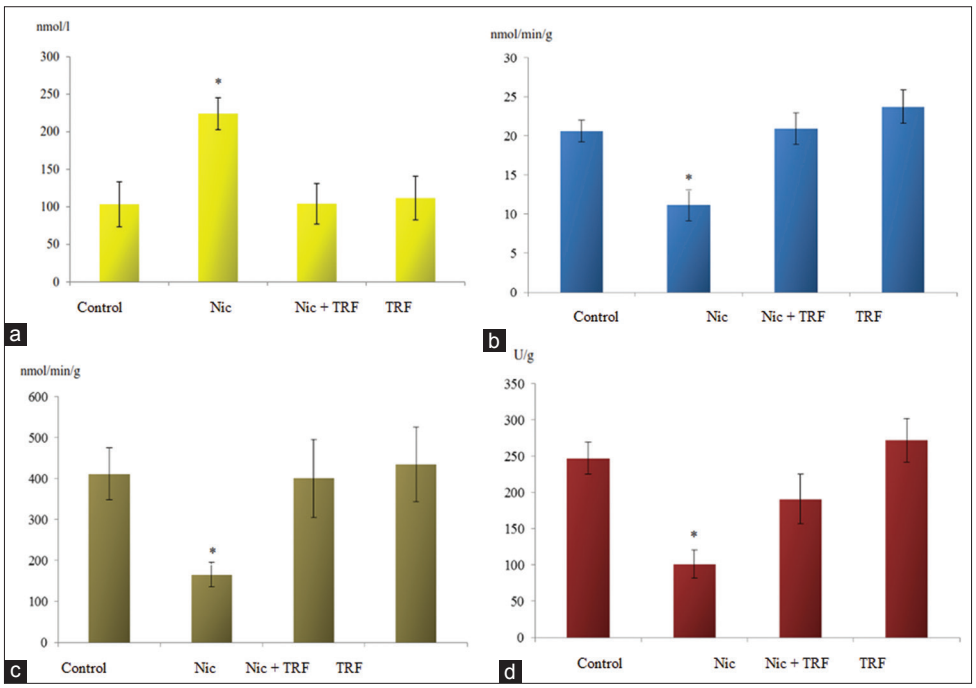

Fig. 1: (a) Plasma malondialdehyde; (b) catalase; (c) glutathione peroxidase; and (d) superoxide dismutase in animals across all groups. (*) indicates significant value at $p<0.05$ when compared to control

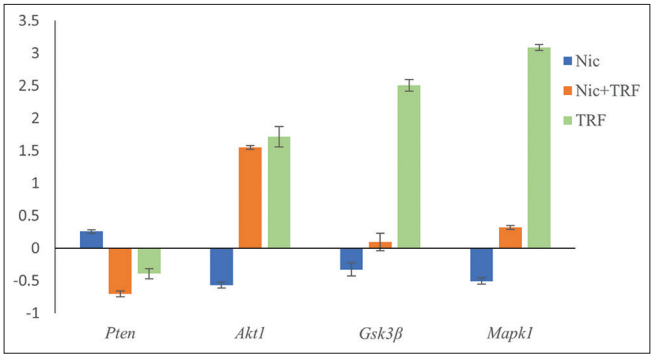

Fig. 2: Gene expression analysis of embryos at 2-cell stage in four different groups; control (C), Nicotine (Nic), Nic+tocotrienol-rich fraction (TRF) (Nic+TRF), and TRF. The comparison of fold change values between *Pten, Akt1, Gsk3β,* and *Mapk1* genes plotted as a bar chart. (*) indicates a significant value in fold change at p<0.05 when compared to control group

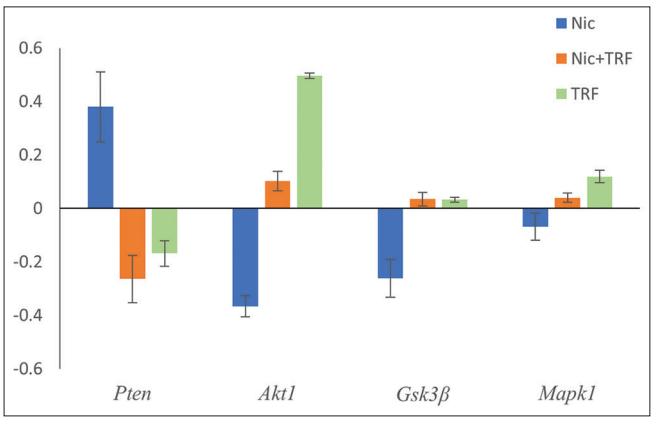

Fig. 3: Gene expression analysis of embryos at 8-cell stage in four different groups; control (C), Nicotine (Nic), Nic+tocotrienol-rich fraction (TRF) (Nic+TRF), and TRF. The comparison of fold change values between *Pten, Akt1, Gsk3β,* and *Mapk1* genes plotted as bar chart. (*) indicates a significant value in fold change at p<0.05 when compared to control group

Nic as 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 effect 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 and 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.

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AUTHORS' CONTRIBUTIONS

All authors contributed equally to the completion of this paper. Nurul Hamirah Kamsani conceived the idea, collected sources of information, initiated, and contributed to manuscript writing. Mohd Hamim Rajikin, Shareniza Ab-Rahim, Yuhaniza Shafinie Kamsani, and Nor Ashikin Mohamed Noor Khan contributed in conceiving the idea, manuscript analysis and review; as well as manuscript writing.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest to disclose in this study.

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