JOURNAL OF CLINICAL AND HEALTH SCIENCES

REVIEW

Tocotrienols and Oxidative Stress in Oocytes and Developing Embryos

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Received
14th April 2017
Received in revised form
14th May 2017
Accepted
22nd December 2017

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ABSTRACT

This review summarizes the impact of tocotrienols (TCTs) as antioxidants in minimizing oxidative stress (OS), particularly in embryos exposed to OS causing agents. OS level is increased, for example, by nicotine, a major alkaloid content in cigarette, which is also a source of exogenous reactive oxygen species (ROS). Increased nicotine-induced OS increases cell stress response, which is a common trigger leading to embryonic cell death. Having more profound anti-oxidative stress effects than its counterpart tocopherol, TCTs improve blastocyst implantation, foetal growth, pregnancy outcome and survival of the neonates affected by nicotine. In reversing cell developmental arrest caused by nicotine-induced OS, TCTs enhances PDK-1 expression in the P13K/Akt pathway and permit embryonic development beyond the 4-cell stage with the production of more morulae. At the cytoskeletal level, TCTs increase the number of nicotine-induced apoptotic cells, through caspase 8 activation in the mitochondria. TCTs facilitate rough endoplasmic reticulum (rER) stress-mediated apoptosis and autophagy, resulting from nicotine-induced OS. Reduced vesicular population in TCT supplemented oocytes on the other hand may suggest reduced secretion of apoptotic cell bodies thus probably minimizing vesicular apoptosis during oocyte maturation. Further extensive research is required to develop TCTs as a tool in specific therapeutic approaches to overcome the detrimental effects of OS.

KEYWORDS: Nicotine, oxidative stress, tocotrienols, antioxidant, mitochondria, rough endoplasmic reticulum, vesicle.

INTRODUCTION

Tocotrienols as antioxidants

Vitamin E, known as vitamin for fertility, includes two groups of closely related fat-soluble compounds, the tocopherols (TCP) and tocotrienols (TCT). Each consists of four isomers α, β, γ and δ. Studies concerning TCT in the beginning were few compared to TCP, where of all papers listed in PubMed, less than 1% relate to TCT and this despite its discovery just a few years after the discovery of TCP. TCP was discovered earlier in 1928 and TCT was discovered a few years later in 1946. The lower publications might be due to its lower availability in nature and therefore not easily available for research studies [1, 2]. Over the years, attention was therefore mainly focused on TCP and substantial amount of money and time was invested in its exploration, development, application and commercialization. However, recent notable discoveries concerning TCT in over 900 published research articles in PubMed have established that this micronutrient, found abundantly in palm oil and barley, provides some health benefits not offered by TCP. Remarkably, TCT isomers have been shown to have more profound anti-oxidative, cholesterol lowering and anti-cancer effects than TCP [3-5]. Consequently, more investigators are now beginning to examine the benefits of TCT. In addition, the availability of purer and isolated forms of TCT is greatly enhancing work on TCT.

Tocotrienols and tocopherols in embryo development

Although TCT is recognized as a more potent cholesterol-, triglyceride-lowering, and chemo-protective agent than TCP, majority of the studies on TCT have focused on its anti-oxidative properties in the prevention of cancer, cardiovascular diseases, bone health and cholesterolemia. Its greater antioxidant activity has been accredited to the presence of 3-*trans*

double bonds, which enhances its incorporation into cell membranes; thereby providing a better lipid-phase antioxidant potency [3, 5]. In reproduction, studies on female mice suggest that TCT enhances fertility even better than TCPs as indicated by marked increments in the number of trophoblasts, improved placentation [4,5], and increased survivability of embryos [3,6]. Indeed, a later study confirmed a better fertility restoration effect of α-TCT in tocopherol transfer protein (TPP)-knockout mice, compared to that of α-TCP [6, 7] This was possibly mediated by transporter Niemann-Pick C1-like 1 via active transport [8-10].

From oocytes to neonates: effects of oxidative stress

Free radicals, such as reactive oxygen species (ROS) are generated as by-products of cellular metabolism. Common forms of ROS include superoxide (O2-), hydrogen peroxide (H_2O_2) and nitric oxide (NO). These carry out important cellular functions under physiological conditions, e.g., immunity/inflammation, signaling/feedback, oxygen sensing, cell proliferation and differentiation. However, ROS are capable of inflicting significant damage to cell structures when they increase to pathological levels. As they can be highly toxic molecules, their levels are normally regulated by the endogenously produced ROS-scavenging enzymes such as glutathione peroxidase (GPx), catalase (CAT) and superoxide dismutase (SOD) [11, 12] or exogenously derived micronutrients such as vitamin C, carotenoids, polyphenols and vitamin E, including tocotrienols (TCTs) [5, 13]. Oxidative stress (OS) arises when there is an imbalance between pro-oxidants and antioxidants, which consequently impairs intracellular homeostasis. The damage from oxidative stress can include damage to cytoskeletal ultrastructures in normal cells [14, 15], in pre- and post-implantation embryos, where it could affect their subsequent development [16-19]. It could also affect intrauterine foetal growth [19, 20], pregnancy outcome and survival of the neonates in mice [21, 22].

Several reports on pre-implantation embryos subjected to ROS and subsequent supplementation of antioxidants are readily available [17, 23, 24]. It should be noted that ROS is capable of inflicting significant damage at the level of the oocyte, and the damage can be translated into impaired embryonic development. Unfortunately, to date, reports regarding ROS and TCT supplementation on the unit that controls cell development, namely cytoskeleton, are still scanty.

Tocotrienols in nicotine-induced oxidative stress in embryo and cellular development

Nicotine addiction during pregnancy has been shown to decrease offspring birth weight [25, 26]. Decreased birth weight in the offspring is one of the factors contributing towards infant death in the world. Effects of nicotine from cigarette smoke are not only limited to active smokers but are evident in passive smokers too. Higher concentration of cotinine in fetal blood exceeding maternal blood concentrations by 15% suggests that nicotine in second-hand smoke concentrates in the foetus [26, 27]. Nicotine-induced embryonic malformation includes open neural tube in the anterior brain regions [28, 29], embryonic retardation and reduced rate of embryo cleavage [23, 30]. At the cellular level, nicotine increases intracellular free calcium in endoplasmic reticulum (ER); an important ultrastructure involved in protein and lipid synthesis of a cell, consistent with increased ER stress response and embryonic cell death [24, 29, 31]. Nicotine also alters the ultrastructure of oocytes [24, 29, 31] and possibly trigger the inflammatory pathways [29, 32].

Recent studies have shown that TCT reduces, perinatally, nicotine-induced adverse effects on blastocyst implantation, foetal growth, pregnancy outcome and survival of the neonates in mice [2, 23]. Maintenance of pregnancy until term in TCT supplemented mice might have also resulted from its ability to sustain the plasma ratio of progesterone : estrogen [23]. Supplementation of 60 mg kg^{-1} day^{-1} of δ-TCT to Balb/c mice produced high number of morulae, evidently through increased expression of 3-phosphonositide-dependant protein kinase-1 (PDK-1) and decreased expression of Akt1 [33]. PDK-1 is involved in cell proliferation, growth, development and apoptosis, mediated by P13K/Akt pathway [34, 35]. This pathway was earlier reported to be inhibited in pre-implantation embryos from mice perinatally treated with 3.0 mg $kg^{-1}day^{-1}$ nicotine, resulting in arrested development of cleavage embryos at 4-cell stage [33]. The proposed mechanisms by which δ-TCT, through expression of PDK-1, allowed pre-implantation

embryos to overcome the developmental blockage were; 1) possibly through enhanced cell proliferation in the absence of activated Akt1, a mechanism known as Akt1-independent signaling OR, 2) through the down-regulation of Akt1, which causes increased level of PIP3, a second messenger in PI3K/Akt pathway [33].

I) Tocotrienols protect against oxidative stress-induced mitochondrial dysfunction

i) Integrative functions of mitochondria

Mitochondria are the major generators of energy in majority of eukaryotic cells, through their generation of ATP. During embryonic development, mitochondria in the syncytiotrophoblast have additional principal functions as the location of synthesis of steroid hormones, apart from being involved in the metabolism and transport of cholesterol [36]. During differentiation of villous cytotrophoblast cells, prior to fusion with the covering syncytiotrophoblast, the mitochondria undergo several morphological changes, including becoming smaller and more rounded. These changes are associated with increased density of mitochondria, which is important in maintaining mitochondrial homeostasis [36, 37].

ii) Mitochondria as a 'source' of reactive oxygen species

Mitochondria, although mostly known as the cell 'powerhouses', are surprisingly the most important physiological and pathological sources of ROS in most types of mammalian cells, both *in vivo* and *in vitro* [38, 39]. They are also a major site and contributors to OS. The generation of ROS in the mitochondria is necessary for redox intracellular signaling in a cell but when produced in excess it contributes to oxidative damage in a number of pathological conditions. Information on how mitochondria produce ROS, began with the demonstration of production of hydrogen peroxide (H_2O_2) by isolated mitochondria [40, 41], which was later confirmed to arise from superoxide ($O2.^-$) dismutation [42, 43]. Superoxide radicals are reported to be generated through electron transfer onto molecular oxygen, mediated by complexes I and III of the electron transport chain [44, 45]. There are three instances proposed to explain the H_2O_2 efflux from the mitochondria. The first is when the NADH/NAD+ ratio is high in the matrix [46, 47]. The second is when CoQ (coenzyme Q) levels are severely reduced, associated with membrane potential-pH gradient imbalance in the electron transport chain, coupled with absence of ATP synthesis [39, 48]. The third instance applies whenever mitochondria are working normally to produce ATP to sustain other functions such as thermogenesis. In the latter instance however, H_2O_2 efflux from mitochondria is negligible as opposed to the earlier instances [39].

iii) 'ROS-induced ROS release' phenomenon: mitochondrial triggers of cellular senescence

The 'cross-talk' between ROS and the intracellular sites of ROS production has been intensively studied [42, 48, 49]. ROS appear to be the important signaling molecules that trigger mitochondrial permeability transition pore (mPTP) induction in each mitochondria within intact cell systems to further stimulate ROS generation. This positive feedback mechanism has been termed as "ROS-induced ROS release" (RIRR). Variety of observations support RIRR as the common mechanism for subsequent ROS generation and amplification [43, 48, 50].

Mitochondria have been identified as not only the source but also the target of the generated ROS, leading to a loss in mitochondrial function, resulting in apoptosis and/or cellular injury [42, 48, 49]. Within oocytes, the role of ROS in compensating mitochondrial distribution is seen in the high abundance of mitochondria. This suggests of a compensatory mechanism by mitochondria to overcome the increased utilization of ATP e.g. during increased oocyte demand [24].

Within ROS-induced mitochondria, reduced or absence of cristae junctions (CJ) from the mitochondrial matrix suggests of CJ remodeling [50-52]. Remodeling of cristae junction facilitates mitochondrial release of cytochrome c, causing apoptosis cascade. The mediator for this event is identified as optic atrophy 1 factor (OPA1), a factor responsible in causing mitochondrial swelling and organelle rupture [51-53]. Other mPTP inhibitors such as CsA, BGK and Ru360, have been identified to reduce percentage of cristae cells [54, 55]. The increased permeability of inner mitochondrial membrane allows for more protons, ions and solutes (up to 1.5 kDa in size) to pass through, consequently

affecting the chemio-osmotic function of mitochondria [39, 50].

Supplementation of 60 mg/kg bw/day TCT produced fewer mitochondria, more dense matrix and increased presence of cristae in 5.0 mg/kg bw/day nicotine-induced mice oocytes [24]. This shows the benefits of TCT in reversing the oocyte compensatory mechanism. Lesser amount of mitochondria within oocyte could probably be linked to depleted ROS level within mitochondria and reversed RIRR mechanism. Whilst excessive ROS can reduce mitochondrial homeostasis and the developmental potential of oocytes in female mice, supplementation of TCT has been shown to increase the contents of antioxidant enzymes, such as SOD1, CAT and GPx [23], and increased expression of anti-apoptotic BCL-XL gene [56]. On the other hand, decreased levels of MDA in oocytes [24] and pre-implantation embryos [57], as well as decreased cytochrome-c and caspase-9 [58], caspase-3 and Bax [56], indicate that TCT may reduce OS and apoptosis at both oocyte and embryonic level, in a way, increasing the development potential of the embryos.

II) Tocotrienols protect against oxidative stress-induced rough endoplasmic reticulum dysfunction

i) Rough endoplasmic reticulum as protein generator in a cell

Rough endoplasmic reticulum (rER) is an organelle, consisting of a network of membranes found all over a cell. Best known as the site of synthesis and post-translational processing of proteins [36] destined for insertion into the cell membrane, its size and structure vary from cell to cell depending on the cell's function. A large quantity of rER is found in cells that synthesize and release a lot of proteins. Large amounts of rER is packed within the syncytiotrophoblasts of placenta, ensuring active ionic pumping, steroid and peptide hormone synthesis and active amino acid transport as part of a dynamic function of placenta [59] essential in maintaining pregnancy and normal fetal development [56]. Additional roles of rER include major intracellular reservoir of calcium, and the site of loading of peptides [60, 61].

To meet the regulation of calcium transport, rER is densed with calcium transporters and ion channels. Flux of calcium triggers tricarboxylic acid (TCA) activity, stimulates mitochondrial electron transport chain and thus adenosine triphosphate (ATP) production as part of protein synthesis [60]. rER maintenance of intracellular calcium homeostasis is modulated through active transport function of the sarco/endoplasmic reticulum calcium ATPase (SERCA) [61]. To facilitate protein folding and maturation, rER chaperone proteins such as Binding Immunoglobulin Protein (BiP) depend on high levels of calcium.

ii) Rough endoplasmic reticulum and oxidative stress

Homeostasis in the rER may be disrupted through various insults, such as elevated expression of proteins that transit the endomembrane system, pharmacological perturbation, genetic mutation of ER chaperones or alterations in calcium or redox status, or viral infection [62]. Recent findings have focused on rER due to its involvement in ultrastructural stress. It has been documented for some time that under increased OS, rER in oocytes appears denser, and increased in size, consistent with enlarged cisternae [24]. Cellular stress is also produced through shorter electron transport chain in rER, which is responsible for 25% of OS in cells [62, 63]. OS also increases calcium release from rER through the ryanodine receptors and inositol-1, 4, 5 triphosphate (IP3R), with the ions being taken up by the mitochondria to facilitate further OS generation [64]. Affected calcium homeostasis impairs the protein folding machinery, leading to accumulated misfolded or unfolded proteins and sequester BiP inside the lumen of rER, thereby elevating the intracellular levels of OS in rER [65]. Several reports exist in the literature describing the increased BiP levels due to OS [63, 65, 66]. The rER must therefore be capable of stress-adapting responses. Failing to do so might result in diverse downstream effects. One of the responses include the activation of intracellular signaling pathway, known as the unfolded protein response (UPR) in the lumen of rER, as a result of unfolded or misfolded protein accumulation [67].

The anti-oxidant properties of TCTs may be responsible for the improvement of embryonic ultrastructures in females with OS-related reproductive dysfunction. Supplementation of 60 mg/kg bw/day of TCTs to OS-induced oocytes maintains the shape of

oocyte with intact zona pellucida (ZP), reduces the density and enlargement of OS-induced rough endoplasmic reticulum (rER) comparable to that in normal oocytes [24]. The novel mechanism by which this potent antioxidant activates OS response in rER however was only discovered recently. The decreased accumulation of unfolded proteins in TCTs supplemented rER reduced the size of rER cisternae [67]. Enlarged cisternae have been associated with the enlargement of rER; a feature associated with perturbed intracellular redox homeostasis. Another study optimized 40 μM of γ-TCT as the concentration that induces autophagy by inducing ER stress-mediated apoptosis and releasing Ca^{2+} ions. This is marked by increased content of autophagy biomarker Beclin 1 [68]. Taken together, TCTs may facilitate concurrent action of rER stress-mediated apoptosis and autophagy, in order to provide cellular protection on rER as a result of nicotine-induced OS. This may explain the reduced number of rER in the 60 mg/kg bw/day TCT treated oocytes reported previously [24].

III) Tocotrienols protect against oxidative stress-induced vesicular dysfunction

i) Vesicles carry supplies for cell growth

Vesicles are the "cargo transporters" of a cell, transporting and containing nutrients, metabolites and nucleic acids that provide the essentials to the growing cells. All these components are imported into a cell from external fluids through diffusion, endocytosis, or via cumulus complex gap junctions. They are stocked in the vesicles before being delivered, digested or secreted within or outside of the cell [69]. The size of a vesicle may vary between 30 and 3,000 nm in diameter. Classed under several types, their main function is to transport biochemical signal molecules depending on the biomolecular composition, cell sources and conditions [70]. The main transportation process involves clathrin-mediated-endocytosis, an endocytosis by inward budding of plasma membrane vesicles [71].

The relationship between vesicular chemical transportation and population appears to be related to OS. Previous studies have shown that when subjected to extracellular oxidative stress, vesicular exocytosis may be hampered. This is documented as a decreased quantal release consistent with decreased synaptic response; a response crucial for neurotransmitter release [72, 73]. As a result, vesicular catecholamine concentrations (mainly neurotransmitters norepinephrine and epinephrine) are reduced, thus reducing also the amount of neurotransmitter release during exocytosis. The subsequent events include vesicular volume shrinkage, and reduced volume of cell cytoplasm resulting in interrupted vesicular fusion events [74, 75]. Another study has shown that the vesicular uptake by the ovarian cells was inhibited by chlorpromazine, an antipsychotic medication [76]. There was also another study that reported otherwise [24]. The number of vesicles when treated with 5.0 mg/kg bw/day nicotine was found to multiply, as a result of the compensatory mechanism required by the vesicles to transport excess pro-oxidants, apoptotic cells, damaged metabolites and nucleic acids. A recent study by Martinez et al. (2015) however suggests no correlation between cellular stress and vesicle size or concentration [77]. Depending on their biological functions and cargos, OS induced-vesicles may be immunosuppressive, or may increase in number and density to recycle pro-oxidants, apoptotic or necrotic material. Changes in the balance of vesicular formation and distribution in a cell may therefore be central to a compromised cell integrity.

In the TCT-supplemented oocytes induced by OS, transmission electron microscopy (TEM) revealed less number of vesicles, similar to that in the control group. The energy stock-containing vesicles appeared to be fewer, more translucent and seem to be "resorbed" in the ooplasm, making them not very prominent [24]. This suggests that TCT altered the secretory function of the vesicles, thus minimal disposal of apoptotic cell bodies from the cell through exocytosis, consistent with decreased rate of cell death. Decreased abundance of vesicle in TCT supplemented oocytes suggests functional repair by TCT through less redistribution of phospholipids and repositioning of phosphatidyl serine to the outer leaflet of the plasma membrane, and also less contraction of the actin–myosin machinery by the activation of myosin light chain kinase; all of which are important events in cell apoptosis distinct pathway [77-79]. Therefore, reduced vesicular population in TCT supplemented oocytes probably reduce the secretion of apoptotic cell bodies, minimizing the impact of OS in

the vesicles during oocyte maturation. However, more research data is required to explain the distribution of TCT-supplemented OS-induced vesicles before conclusive evidence describing the effectiveness of TCT on the vesicles could be provided.

IV) Tocotrienols trigger major apoptosis pathway involving caspase-8 activation

Numerous reports have also linked TCTs with cell morphological features related to OS induced-apoptosis [55, 80, 81]. Human lung and brain cancer cell lines, for example, when treated with 3μM β-TCT for 24 h, showed nuclear chromatin condensation, equivalent to mid-apoptosis. While at 48 h after treatment, almost all cells were dead [81]. γ-TCT similarly enhances anti-proliferative action against human breast cancer cells, with evidence of chromatin condensation including pre-apoptotic and apoptotic nuclei [82] that lead to the formation of apoptotic bodies [82, 83]. Caspase-mediated apoptosis in most cells is known to activate either the death receptor (extrinsic) pathway or mitochondrial (intrinsic) pathway, or both pathways. Significantly enhanced caspase-8 activities in β-TCT-treated human lung and brain cancer cells suggest that both pathways are activated [81]. Extrinsic pathway engages the death receptors on the cell membrane and recruits the adaptor protein and procaspase-8 to form a death-inducing signaling complex. This is then followed by auto cleavage and activation [84, 85]. Procaspase-8 proteins undergo auto cleavage and activation. Active caspase-8 then activates caspase-3, regulated by the abundance of apoptosis inhibitors. The intrinsic pathway, on the other hand, involves an induction of mitochondrial permeability transition. This leads to cytochrome c release, caspase activation and recruitment domain (CARD) adaptor protein, procaspase-9 and apoptotic protease activating factor 1 (APAF-1) accumulation in cytosol and apoptosome, resulting in caspase-9 activation. This ultimately activates effector caspases such as caspase-3 [85, 86]. Caspase 8 also truncates BID, a pro-apoptotic member of the Bcl-2 protein family, causing cytochrome c release from mitochondria into cytosol. The truncation then initiates further downstream effector caspases activity [58]. The final outcome will be the apoptotic cell death of the TCT treated-cancer cells [81], consistent with decreased density of TCT treated nicotine-induced mitochondria as reported previously [24]. Nevertheless, there are also some contradicting reports that question the role of caspases and modulation of Bax/Bcl-2 in the TCT executed apoptotic pathway [87, 88]. However, the possibility that TCT might exhibit a different cellular mechanism of apoptosis in different cell types induced by different kinds of ROS warrants further investigation.

CONCLUSION

Oxidative stress does not only impair cellular integrity of pre-and post-implantation embryonic development, intrauterine foetal growth, pregnancy outcome and survival of the neonates, but also the cytoskeletal stability of mitochondria, rER and vesicles of oocytes. This review identifies potential mechanisms of TCTs that might be responsible in reversing OS-induced cytoskeletal toxicity in oocytes and perhaps other cells. Systematic investigations into the molecular potentials of TCTs in combating OS, not only on the oocytes, as discussed in this review will reveal the fascinating ways of TCTs in targeting the specific genes through specific signaling pathways. Such initiation is important due to the scarcity of molecular information pertaining to the variable influence of spermatozoa on sperm-oocyte interaction, which ultimately will dictate embryonic development. Only through more extensive research, the development of TCT as a specific therapeutic approach in overcoming OS induced-oocyte dysfunctions could be initiated.

Conflict of Interest

Authors declare none.

Acknowledgements

The authors wish to thank Faculty of Medicine, UiTM Sungai Buloh Campus for the support in writing this paper.

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