Original article

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Targeting genes in insulin-associated signalling pathway, DNA damage, cell proliferation and cell differentiation pathways by tocotrienol-rich fraction in preventing cellular senescence of human diploid fibroblasts

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Abstract

Background and Objective. Tocotrienols have been known for their antioxidant properties besides their roles in cellular signalling, gene expression, immune response and apoptosis. This study aimed to determine the molecular mechanism of tocotrienol-rich fraction (TRF) in preventing cellular senescence of human diploid fibroblasts (HDFs) by targeting the genes in senescence-associated signalling pathways.

Materials and Methods. Real time quantitative PCR (qRT-PCR) was utilized to evaluate the expression of genes involved in these pathways.

Results. Our findings showed that *SOD1* and *CCS-1* were significantly down-regulated in pre-senescent cells while *CCS-1* and *PRDX6* were up-regulated in senescent cells ($p<0.05$). Treatment with TRF significantly down-regulated *SOD1* in pre-senescent and senescent HDFs, up-regulated *SOD2* in senescent cells, *CAT* in young HDFs, *GPX1* in young and pre-senescent HDFs, and *CCS-1* in young, pre-senescent and senescent HDFs ($p<0.05$). TRF treatment also caused up-regulation of *FOXO3A* in all age groups of cells ($p<0.05$). The expression of *TP53, PAK2* and *CDKN2A* was significantly increased in senescent HDFs and treatment with TRF significantly down-regulated *TP53* in senescent cells ($p<0.05$). *MAPK14* was significantly up-regulated ($p<0.05$) in senescent HDFs while no changes was observed on the expression of *JUN*. TRF treatment, however, down-regulated *MAPK14* in young and senescent cells and up-regulated *JUN* in young and pre-senescent HDFs ($p<0.05$).

Conclusion. TRF modulated the expression of genes involved in senescence-associated signalling pathways during replicative senescence of HDFs. ***Clin Ter 2015; 166(6):e365-373. doi: 10.7417/CT.2015.1902***

Key words: *cellular senescence, genes expression, senescence-associated signalling pathways, tocotrienol-rich fraction*

Introduction

Vitamin E is an effective antioxidant, radical scavenger (1), and has the ability to stabilise cellular membrane (2). It has therefore been examined in numerous *in vivo* and *in vitro* studies. Vitamin E was originally being isolated as tocopherols, which consists of four isomers; α-, β-, δ- and γ- tocopherols. Now, vitamin E is referred as a class of fat-soluble antioxidants consisting of both tocopherols and tocotrienols (3). The differences in biological activities of the two are attributed to their structural differences, which lie in the presence of three double bonds in the farnesyl isoprenoid tail in tocotrienol. Serbinova et al. (1991) (4) reported that the antioxidant activities of tocotrienols rely on the incorporation of the compounds in cell membranes, and the accessibility of the antioxidants to the lipid radicals. Their data suggested that α-tocotrienol distributes more uniformly in membranes and increases fluidity of membrane lipids, whereas α-tocopherol appears to have a greater tendency to cluster in membranes (5). Lately, tocotrienols have been found to exert superior antioxidant (6, 7), anticancer (8), cholesterol-lowering capabilities (9), anti-inflammatory (10) and neuroprotective properties (11). These properties of tocotrienols could be attributed to their ability to modulate various gene expressions (12). Even though tocotrienol has been reported to be more effective than tocopherol, combination of α-tocopherol and all tocotrienol isomers in tocotrienol-rich fraction (TRF) from palm oil extract is more effective as compared to single isomer of vitamin E (13).

Harman (14) reported that formation of free radicals is responsible for diminishing human life span and causes changes that resembled ageing. He then proposed that ageing and age-related diseases might be due to the cumulative oxidative damage which, in turn, is modifiable by genetic and environmental factors (15). At present, the questions arise as what can prolong life expectancy and whether it can be controlled externally, and is there a specific pathway or diet modification that may modulate ageing.

Study of ageing or senescence was first recognized more than 40 years ago during the studies of cultured human fibroblasts by Hayflick and Moorhead (16). They discovered that normal human cells were prevented from growing indefinitely in culture and reached the replicative senescence stage. Senescent cells have a characteristic of enlarged, flattened morphology (7), express senescence-associated β-galactosidase (SA β-gal) (7), and show altered

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gene expression (12). Various genes involved in cell cycle regulation, immune system and inflammation, cytoskeleton, stress response, oxidative status and metabolism are known to be altered during replicative senescence of cells in culture (17) and animal models (18). In animal studies, it was also accounted that 63% of these genes showed a reversal of their expressions by caloric restriction (19).

Cellular senescence can be identified by signalling pathways that are involved in activation of senescence programme. These molecular pathways are associated with energy metabolism, cell cycle arrest, loss of proliferation and oxidative stress response. Inhibition of insulin/insulin-like growth factor-1 signalling (IIS) and its downstream effectors phosphatidylinositol 3-kinase (PI3K) and AKT lead to activation of forkhead box O (FOXO) transcription factor. FOXO has been associated with decrease in energy metabolism and extension of lifespan (20). Markers of senescence cells for cell cycle arrest include tumour protein p53 (*TP53)*, cyclin-dependent kinase inhibitor 1A (*CDKN1A*), cyclin-dependent kinase inhibitor 2A (*CDKN2A)*, cyclin-dependent kinase inhibitor 1B (*CDKN2B*), retinoblastoma gene (*RB*) and checkpoint kinase gene (*CHK*) (21). Mitogen-activated protein kinase 14 (MAPK14) controls senescence growth arrest by activating expressions of TP53-dependent *CDKN2A* and TP53-independent p21 protein (Cdc42/Rac)–activated kinase 2 (*PAK2*) (21). Besides, MAPK14 and c-Jun N-terminal kinases (JNK) act antagonistically to regulate CDKN2A protein expression in stress-induced premature senescence (22). Reactive oxygen species (ROS) levels are increased during replicative senescence (23) and ROS levels also implicated in the induction of replicative senescence (24). Nevertheless, the interplay between cellular antioxidant defence, IIS pathway, DNA damage pathway and cell proliferation pathway in replicative senescence is remained to be elucidated. Thus by using the Hayflick model of ageing, this study was conducted to determine the expression of genes in senescence-associated signalling pathways during cellular senescence and its modification by treatment with TRF. Assessment was done to genes involved in IIS pathway, DNA damage pathway, and cell proliferation and differentiation pathway.

Materials and Methods

Cell culture and the induction of senescence

This research has been approved by the Ethics Committee of Universiti Kebangsaan Malaysia (Approval Project Code: FF-218-2008). The primary human diploid fibroblasts (HDFs) were derived and maintained as described by Makpol et al. (7). Primary HDFs were derived from the foreskins of three 9 to 12 year-old boys post circumcision. Written informed consents were obtained from parents of all subjects. The samples were aseptically collected and washed several times with 75% alcohol and phosphate buffered saline (PBS) containing 1% antibiotic-antimycotic solution (PAA®, Pasching, Austria). After removing the epidermis, the pure dermis was cut into small pieces and transferred into a falcon tube containing 0.03% collagenase type I solution (Worthington Biochemical Corporation®, Lakewood, NJ, USA). Pure dermis was digested in the incubator shaker at 37C for 6–12 h. Then, cells were rinsed with PBS, before being cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% foetal bovine serum (FBS) (PAA) and 1% antibiotic-antimycotic solution at 37°C in 5% CO_2 humidified incubator. After 5–6 days, the cultured HDFs were harvested by trypsinisation and expanded into new T25 culture flasks (Nunc®, Roskilde, Denmark) with expansion degree of 1:4. When the subcultures reached 80–90% confluence, serial passaging was done by trypsinisation and the number of population doublings (PDs) was monitored until HDFs reached senescence. For subsequent experiments, cells were used at either passage 4 (young cell, PD < 12), passage 15 (pre-senescent cell, 30 < PD < 40), and passage 30 (senescent cell, PD > 55).

Preparation of TRF treatment solution

Stock solutions of TRF (Sime Darby Berhad®, Kuala Lumpur, Malaysia) were freshly prepared in 100% ethanol (1:1) and kept at -20°C for not more than one month. Immediately before use, TRF was incubated with FBS overnight at 37°C. Dilution of TRF concentrations was prepared in culture medium mixed with 50% ethanol. Treated HDFs were incubated with 0.5 mg/mL TRF for 24 h while untreated cells were cultured in DMEM containing 10% FBS(7). The media for both control and treated cells were replaced simultaneously, and were harvested on the same day.

RNA extraction

RNA extraction was performed using TRI Reagent® (Molecular Research Center, Cincinnati, OH, USA), according to manufacturer's instructions. Cells were lysed using 1 mL of TRI Reagent® per 10 cm^2 of culture dish area. After RNA precipitation and washing by 75% ethanol, the RNA pellet was briefly air-dried for 8-10 min. Finally, the RNA pellet was dissolved in RNase/DNase-free water and was aliquoted into few tubes to avoid repeated freeze-thawing.

Primer design and optimization

Primers for human glyceraldehyde 3-phosphate-dehydrogenase (*GAPDH),* superoxide dismutase 1 (*SOD1),* superoxide dismutase 2 (*SOD2),* catalase (*CAT),* glutathione peroxidase 1 (*GPX1)*, copper chaperone for superoxide dismutase 1 *CCS-1,* peroxiredoxin 6 (*PRDX6*) , forkhead box O3 (*FOXO3A)*, *CDKN2A , PAK2 , TP53,* MAPK14 and jun proto-oncogene (JUN) (Table 1) were designed with GeneBank database sequences and aligned by BLAST (Basic Local Alignment Search Tool) using the Primer 3 software (25) (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). These genes were amplified using iScript™ One-Step RT-PCR Kit with SYBR® Green (Bio-Rad, Hercules, CA, USA) to a final volume of 25 µL. To optimize the specificity of the primers, the purified PCR products were then quantified by running on a 2% agarose gel prestained with ethidium bromide along with a 100 bp DNA step ladder (Promega, Madison, WI, USA) (Fig. 1).

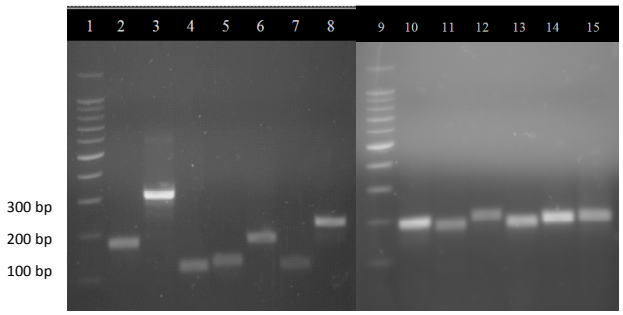

Fig. 1. Analysis of PCR products on 2% agarose gel electrophoresis. Figure shows specificity of primers. Lane 1; 100 bp marker, lane 2; SOD1 primer, lane 3; SOD2 primer, lane 4; CAT primer, lane 5; GPX1 primer, lane 6; CCS-1 primer, lane 7; PRDX6 primer, lane 8; GAPDH primer, lane 9; 100 bp marker, lane 10; FOXO3A primer, lane 11; CDKN2A primer, lane 12; PAK2 primer, lane 13; TP53 primer, lane 14; MAPK14 primer, lane 15; JUN primer.

Real-time quantitative RT-PCR (qPCR)

qRT-PCR reaction was performed with RNA as templates, primers of the targeted genes and iScript™ One-Step RT-PCR kit with SYBR® Green. The amplifications were carried out by iQ5 Multicolor Real-Time PCR system (Biorad) with as follows programme: cDNA synthesis at 50°C for 20 min, iScript reverse transcriptase inactivation at 95°C for 4 min, followed by 38 amplification cycles of denaturation at 95°C for 10 s and 61°C (primer annealing and extension) for 30 s. After the end of the last cycle, melting curve analysis was generated at 95°C for 1 min, 55°C for 1 min and 60°C for 10 s (70 cycles, set point temperature increased after cycle 2 by 0.5°C). qRT-PCR was performed using *GAPDH* as an internal reference to normalise all RNA expression levels (26).

Statistical analysis

Each experiment was carried out in triplicates with at least 3 independent cultures with comparable results. Data was analysed by one-way analysis of variance (ANOVA) followed by post-hoc multiple comparison tests. $p \leq 0.05$ was considered statistically significant.

Results

Expression of antioxidant-associated genes

SOD1 and *CCS-1* were significantly down-regulated in pre-senescent cells as compared to young cells ($p<0.05$) (Fig. 2). However, *CCS-1* was up-regulated in senescent cells as compared to pre-senescent HDFs ($p<0.05$). *PRDX6* was significantly up-regulated in senescent cells as compared to young and pre-senescent cells ($p<0.05$). Treatment with TRF significantly down-regulated ($p<0.05$) *SOD1* in pre-senescent and senescent cells and up-regulated *SOD2* in senescent cells as compared to untreated control ($p<0.05$). TRF treatment also significantly up-regulated *CAT* in young HDFs, up-regulated *GPX1* in young and pre-senescent HDFs, and up-regulated *CCS-1* in young, pre-senescent and senescent HDFs ($p<0.05$). No significant different was observed on the expression of *PRDX6* with TRF treatment.

Expression of genes in IIS pathway

No significant difference was observed on the expression of *FOXO3A* during replicative senescence of HDFs. TRF treatment, however, caused up-regulation of FOXO3*A* in all age groups of cells compared to untreated control ($p<0.05$) (Fig. 3).

Table 1 Sequences of forward and reverse primers and the product size.

Target gene	Forward primer (5'-3')	Reverse primer (5'-3')	Product size (bp)
GAPDH	TCCCTGAGCTGAACGGGAAG	GGAGGAGTGGGTGTCGCTGT	217
SOD1	GAAGGTGTGGGGAAGCATTA	ACATTGCCCAAGTCTCCAAC	174
SOD2	CGTCACCGAGGAGAAGTACC	CTGATTTGGACAAGCAGCAA	312
CAT	CGTGCTGAATGAGGAACAGA	AGTCAGGGTGGACCTCAGTG	119
GPX1	CCAAGCTCATCACCTGGTCT	TCGATGTCAATGGTCTGGAA	127
CCS-1	ACTTTAACCCTGATGGAGCATCT	AGGTCATCTTCTCCCTCATCAAT	181
PRDX6	CGTGTGGTGTTTGTTTTTGG	TGCTGTCAGCTGGAGAGAGA	120
FOXO3A	GCAAGCACAGAGTTGGATGA	CAGGTCGTCCATGAGGTTTT	186
CDKN2A	AGTGAGGGTTTTCGTGGTTCAC	CCATCATCATGACCTGGTCTTCTA	150
TP53	GGAAGAGAATCTCCGCAAGAA	AGCTCTCGGAACATCTCGAAG	177
PAK2	GATGGCACCAGAGGTGGTTA	TCCCGAAATATTGGGGAAAG	198
MAPK14	GGGGCAGATCTGAACAACAT	GAGCCAGTCCAAAATCCAGA	190
JUN	GTCTACGCAAACCTCAGCAAC	ACTGTCTGAGGCTCCTCCTTC	191

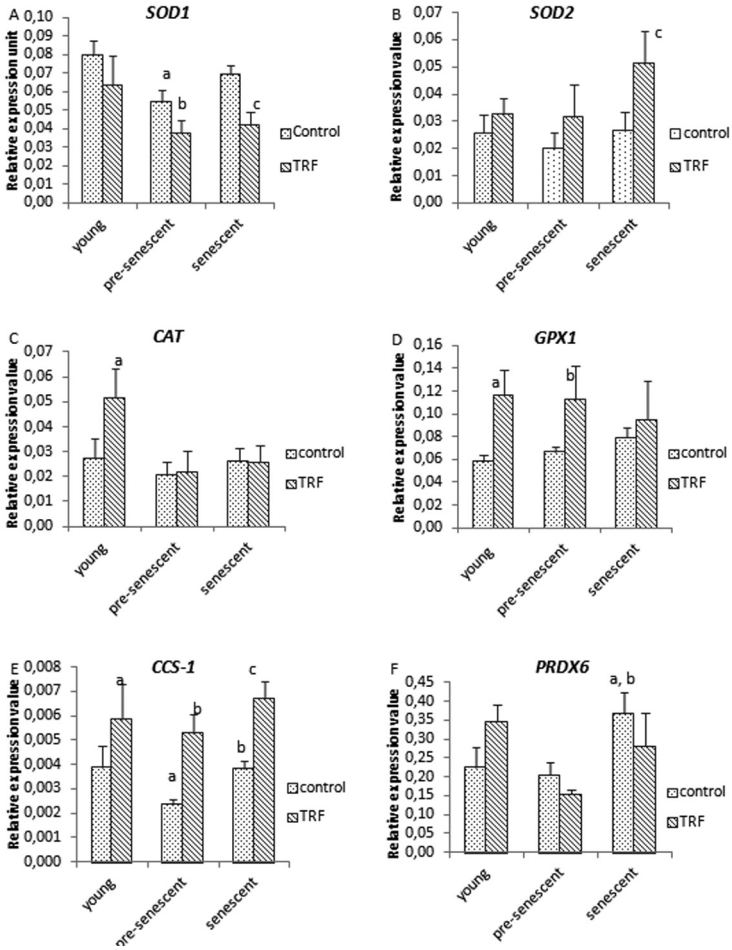

Fig. 2. Effects of replicative senescence and TRF treatment on senescence-associated gene expression. (A) SOD1; (B) SOD2; (C) CAT; (D) GPX1; (E) CCS-1; (F) PRDX6 . Data were expressed as the mean ±SEM, n = 6. [a]p<0.05 compared to control young cells; [b]p<0.05 compared to control pre-senescent cells; [c]p<0.05 compared to control senescent cells.

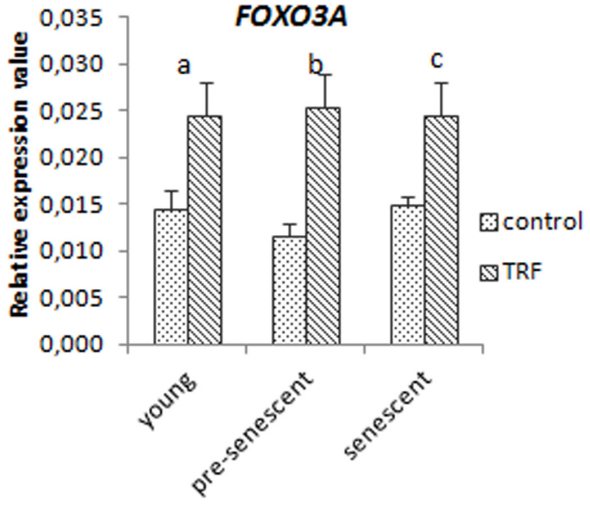

Fig. 3. Effect of replicative senescence and TRF treatment on IIS pathway-associated gene expression, FOXO3A. Data were expressed as the mean ± SEM, n = 6. [a]p<0.05 compared to control young cells; [b]p<0.05 compared to control pre-senescent cells; [c]p<0.05 compared to control senescent cells.

Expression of genes in DNA damage-associated signalling pathway

The expression of *TP53* was significantly increased in senescent HDFs as compared to young and pre-senescent cells (p<0.05) (Fig. 4). *PAK2*[1] and *CDKN2A* were significantly up-regulated in senescent HDFs as compared to pre-senescent cells (p<0.05). Treatment with TRF significantly down-regulated *TP53* in senescent cells (p<0.05) whereby no effect was observed in *PAK2* and *CDKN2A* expressions.

Expression of genes in cell differentiation and proliferation pathway

MAPK14 was significantly up-regulated (p<0.05) in senescent HDFs while no changes was observed on the expression of *JUN* with cellular senescence of HDFs (Fig. 5). TRF treatment, however, down-regulated *MAPK14* in young and senescent cells and up-regulated *JUN* in young and pre-senescent HDFs as compared to untreated control (p<0.05).

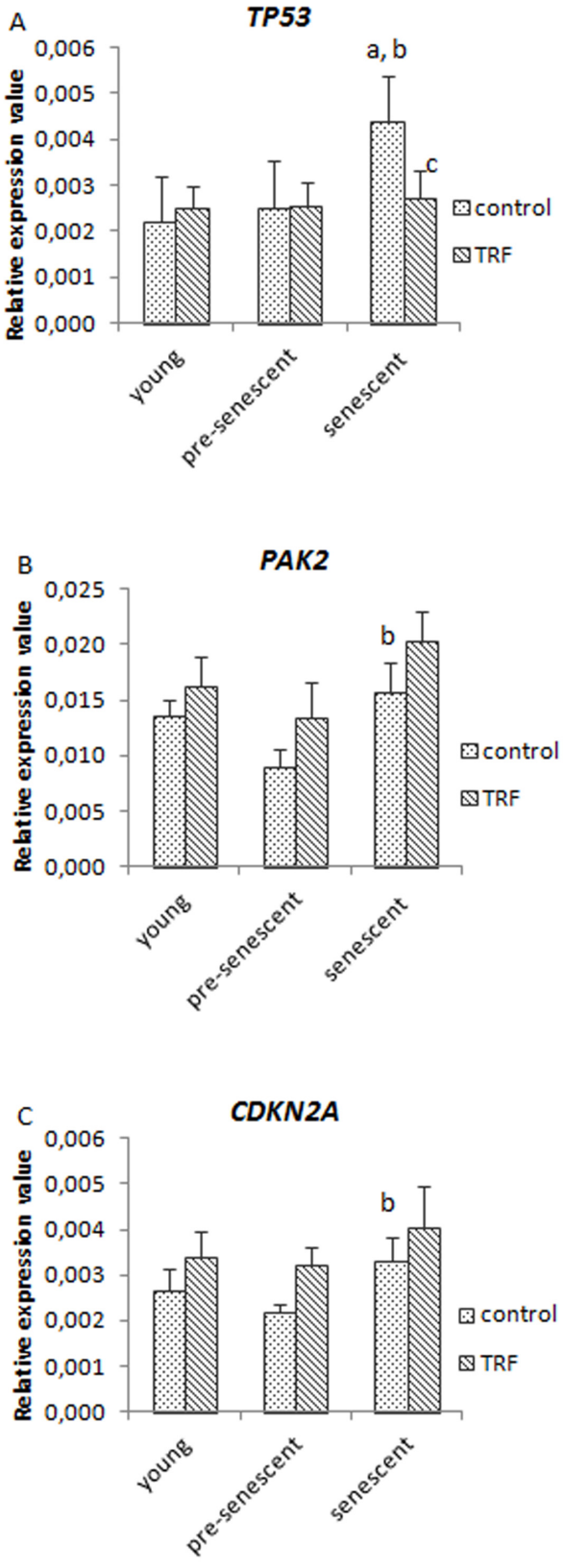

Fig. 4. Effects of replicative senescence and TRF treatment on DNA damage-associated gene expression. (A) TP53; (B) PAK2; (C) CDKN2A. Data were expressed as the mean ± SEM, n = 6. [a]p<0.05 compared to control young cells; [b]p<0.05 compared to control pre-senescent cells; [c]p<0.05 compared to control senescent cells.

Discussion

Cells are equipped with an impressive battery of antioxidant enzymes which protect the balance of intracellular oxidative status. The enzymes are involved in the removal of superoxide in the form of hydrogen peroxide by SODs, and the conversion of hydrogen peroxide to water by the action of CAT and GPX1 (27). The balance between the first and second actions are critical to prevent accumulation of superoxide which is toxic to macromolecules, and accumulation of hydrogen peroxide intermediates that can produce ROS is more dangerous than superoxide (Fig. 6).

Our results showed that in cellular senescence, the expression of *SOD1* was decreased in pre-senescent cells while no significant change was observed for *SOD2*, *CAT* and *GPX1*. These findings are in line with findings reported by Hazane et al. (28) using cDNA microarray which showed unaltered expressions of the antioxidant genes during ageing of primary human fibroblasts in three age groups. However, in other studies, expressions of *SOD1, SOD2* and *CAT* were increased during continuous ageing of fibroblasts in males and females compared to young cells (29), and reduction of *SOD* and *GPX* expressions with increasing age (30). Modification of the antioxidant status during ageing is still unclear and is probably through a complex regulation. Evidence of antioxidant enzymes involvement in maintaining human health is not strong since genetic variation for most genes have not been studied in such a relationship.

Our findings showed that in cellular senescence of HDFs, the relative expression value of *GPX1* was almost similar to the relative expression value of *SOD1*, suggesting that the rate of decline in superoxide coincides with the transition rate of hydrogen peroxide Another important protein in maintaining redox balance in the cells is CCS-1 which has been proposed as a sensitive and specific biomarker to the status of copper ions and delivery of copper ions to the SOD1 (31). In this study, down-regulation of *CCS-1* was found to be in tandem with the decline of *SOD1* in pre-senescent and senescent cells, indicating its function in the activation of mammalian SOD1 enzyme.

Although the effects of tocotrienol on cancer-related gene expressions have been studied extensively, limited data are available regarding the role of tocotrienol in modulating senescence-associated gene expressions. From our data analysis, *SOD1* was found to be down-regulated whereas *SOD2, CAT* and *GPX1* were up-regulated with TRF treatment. Reduction of *SOD1* expression in this study may not influence the antioxidant status or cellular senescence as several other genes are also involved in this pathway. It also may explain the antioxidant function of tocotrienol to combat superoxide, thus no action of SOD1 enzyme occurs. In our previous study, we found that tocotrienol has the potential as anti-ageing entity which compensated the role of antioxidant enzymes in cellular ageing of HDFs (32).

Previous *in vivo* study showed increased concentrations of glycogen, SOD, CAT and GPX in the liver, and increased concentrations of glycogen and SOD in the muscle tissues of mice supplemented with TRF (33). The finding concluded that TRF improved the physiological conditions and reduced oxidative stress induced by physical activity. However, the specific isozyme of SOD was not determined. γ-Tocotrienol

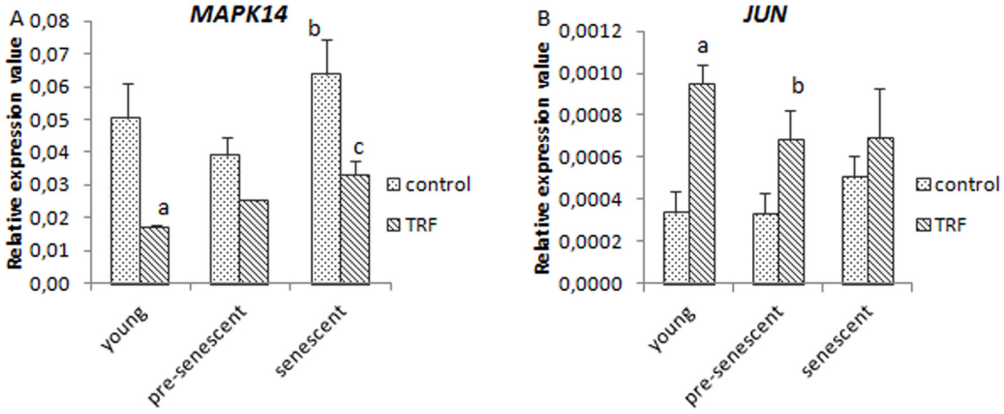

Fig. 5. Effect of replicative senescence and TRF treatment on cell differentiation and proliferation pathway-associated gene expression. (A) MAPK14; (B) JUN. Data were expressed as the mean ± SEM, n = 6. [a] p<0.05 compared to control young cells; [b]p<0.05 compared to control pre-senescent cells; [c]p<0.05 compared to control senescent cells.

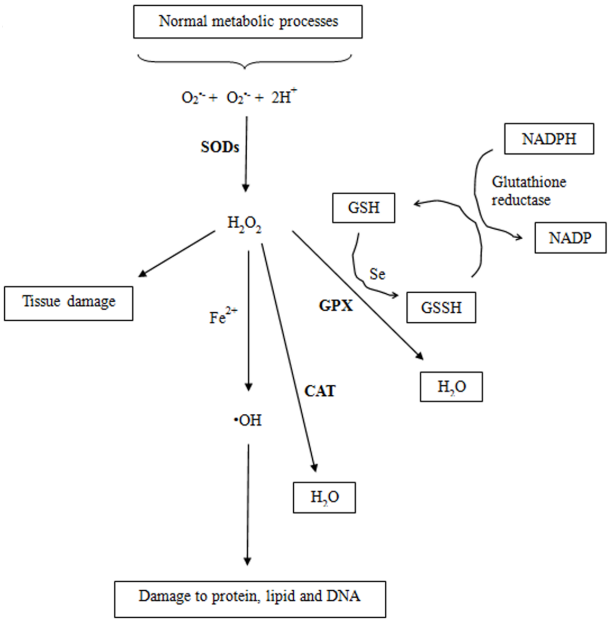

Fig. 6. Endogenous antioxidant reaction mechanism.

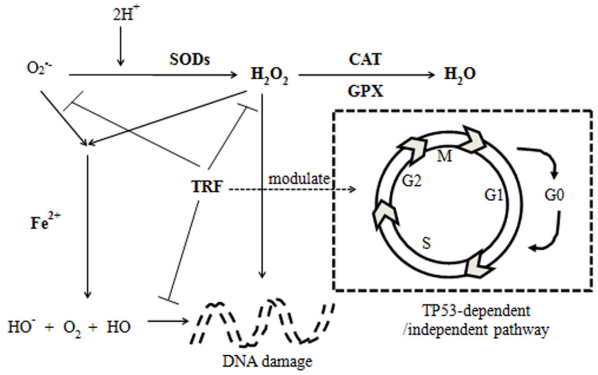

Fig. 7. TRF action in preventing free radical formation and DNA damage.

may also prevent increased blood pressure, reduce lipid peroxides in plasma and blood vessels, and improve antioxidant status by increasing the enzymatic activity of SOD (34). Up-regulation of some antioxidant-associated genes expressions by TRF treatment in this study may be caused by the non-antioxidative function of tocotrienol. The potential of tocopherols as a gene regulator particularly protein kinase C has been suggested in some studies (35, 36). Therefore, TRF may directly and indirectly control the redox status by scavenging free radicals and superoxide as well as by regulating genes, whose products influence ROS generation.

PRDX6 enzyme is also a peroxide scavenger similar to GPX1 but PRDX6 is capable of reducing phospholipid hydroperoxides (37). Mice lacking *PRDX6* gene showed a high rate of lipid peroxidation, concurrent with the increase of oxidative stress. It was shown that with decreased phospholipid peroxides by PRDX6, peroxidation of cell membranes was prevented (37). Therefore, up-regulation of *PRDX6* expression in senescent cells could lead to a reduction in the cellular phospholipids peroxide activity.

FOXO3A is a transcription factor that regulates IIS pathway to influence mammalian metabolism and longevity (38). Genetic variation of *FOXO3A* correlated with human lifespan (39), whereby the down-regulation of FOXO3A protein expression gave rise to senescence phenotypes (40). The present study revealed that young, pre-senescent and senescent cells expressed relatively the same amount of *FOXO3A*. The expression increased when cells were subjected to TRF treatment. Kim et al. (40) have indicated that inhibition of FOXO3A protein induces expression of senescence phenotype on fibroblasts cells. Our findings correlate with a study by Adachi and Ishii (2000) (41) who

examined the protective effects of tocotrienols against oxidative damage using the *C. elegans* model. Tocotrienol prolong lifespan by increasing the resistance of the cell to oxidative stress. In *C. elegans*, DAF-16 (FOXO homologue) enhances the expression of SOD3 enzyme (homologous mammalian SOD2) (42). Further research detected that *FOXO3A* has a consensus site found in the *SOD2* promoter, expecting similarities between the DAF-16 to induce SOD3 mitochondrial enzyme activity. This may also explain the increased *SOD2* expression in senescent cells treated with TRF in this study.

To understand the ageing process as well as to maintain the health and increase lifespan, the pathway that affects the metabolism, maintenance and repair mechanisms of the genes need to be analysed. DNA damage has been hailed as one of the causes of ageing (43). In response to DNA damage, increased expression of TP53 will induce cell cycle inhibition and allow the cells to encounter either repair mechanism or apoptosis (44), by regulating cell cycle arrest at G_1 and G_2/M checkpoints, together with cyclin-dependent protein kinase inhibitor p21. PAK2 is a protein kinase that is activated during DNA damage to induce cell cycle arrest (45). In this study, expressions of *TP53* and *PAK2* were increased during replicative senescence, which agree with the need of the cells in DNA repair.

CDKN2A, a tumour suppressor protein belongs to Cip/Kip family of CDK inhibitor (CDKI), is one of the biomarkers of senescence. It regulates G_1 cell cycle arrest via the pRB pathway (46). Expression of its gene *CDKN2A* increases with age as shown by this study, which correlates with an *in vivo* study (46). In our previous study, CDKN1A which belongs to INK family of CDKI was also increased in senescent cells (47).

MAPK14 (also known as p38 MAPK) (22) and JUN (48) proteins are responsible in regulating replicative senescence. MAPK14 is a mitogen-activated protein kinase (MAPK), involved in various different signalling cascades such as those that regulate inflammation (49) and contact inhibition in fibroblasts (50). JUN is required for cell proliferation where mouse embryo fibroblasts lacking of *JUN* undergo premature senescence due to spontaneous DNA damage accumulation (48). In this study, *JUN* expression was relatively unchanged during cellular senescence, but TRF treatment resulted in increased *JUN* expression in the young and pre-senescent fibroblasts.

In the present study, *CCS-1* was up-regulated in all groups of cells treated with TRF. The non-parallel increment with that of *SOD1* expression may be due to higher sensitivity of *CCS-1* to the concentration of copper in the cell, rather than reduction of SOD activity. CCS-1 protein has been shown to interact directly with SOD1 protein *in vitro* and *in vivo* (51). The loss of *CCS-1* gene caused increased sensitivity to ROS and a significant increase in protein carbonyl levels, lipid peroxidation and genome instability (52). However, CCS-1 is not completely necessary for SOD1 function since SOD1 can supply copper independent of CCS-1 (53). Therefore, the importance of CCS-1 in ageing is probably due to its role in supplying copper to other proteins such as CoxII rather than SOD1 (54).

A study by Tolle et al. (55) found that rats which were controlled by reducing dietary vitamin E (α-tocopherol) showed no change in the concentration of platelet activator factor hydroperoxides and related oxidation products in rat alveolar type II cells. Rats fed with a vitamin E-rich diet resulted in no significant change in the *PRDX6* expression, leading to the conclusion that *PRDX6* expression is not affected by the concentration of vitamin E in the cell. Similarly, results obtained from the present study revealed no significant change in *PRDX6* expression in young, pre-senescent and senescent cells treated with TRF.

Our previous study showed that tocotrienol prevents cell cycle arrest and apoptosis in HDFs (7, 56) which could be attributed to its action in preventing free radical formation and DNA damage by acting as antioxidants while also possessing non-antioxidant function (Fig. 7). In addition, TRF may act directly on the genes that control DNA damage and cell cycle regulation (7, 12, 57).

In the present study, TRF was found to down-regulate *TP53* expression but did not affect *PAK2* and *CDKN2A* expressions in senescent cells. Therefore, cell cycle restrictions in the senescent cells were inhibited to allow cell cycle progression entering S phase. This observation was supported by cell cycle profile analysis which represented an increase in the number of cells entering the S phase and reduce DNA damage when cells were treated with TRF (7, 56). A study by Pinkston-Gosse and Kenyon (58) has proposed that one of the downstream targets of FOXO family is p53. They can bind to each other in response to stress and share some of the targeted genes. Consequently, p53 can also act through IIS pathway. TRF-induced enhancement of *FOXO3A* then modulated *TP53* expression in either the cell cycle or apoptosis pathway.

Many studies have reported the involvement of vitamin E in the modulation of MAPK activation pathway (59-61), thus implicated in the regulation of several events such as cell proliferation, cell differentiation, growth inhibition and apoptosis. A study using Trolox, a vitamin E analogue, showed that vitamin E was able to prevent hydrogen peroxide production in keratinocytes. When high concentration of Trolox was applied in a short time, activation of MAPK14 was prevented effectively (62). Based on that, down-regulation of *MAPK14* expression observed in each group of treated cells in this study may suggest a protective effect by TRF. Treatment with TRF also proved that TRF has the ability to reduce DNA damage and reverse the cell cycle arrest associated with senescence (7).

In conclusion, TRF modulated the expressions of genes involved in IIS pathway, DNA damage pathway, and cell proliferation and differentiation pathway during replicative senescence of HDFs via its antioxidant and non-antioxidant functions. However, further investigations are still required to determine the exact mechanism of TRF action.

Competing interests

The authors declare that they have no competing interests.

Acknowledgements

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