

Food Chemistry
Manuscript Draft

Manuscript Number: FOODCHEM-D-12-01113

Title: Gamma-tocotrienol modulated gene expression in senescent human diploid fibroblasts as revealed by microarray analysis

Article Type: Research Article (max 7,500 words)

Keywords: Gamma-tocotrienol, cellular aging, human diploid fibroblasts, gene expression, microarray analysis

Abstract: The effect of gamma-tocotrienol, a vitamin E isomer, in modulating gene expression in cellular aging of human diploid fibroblasts (HDFs) was studied. Senescent cells at passage 30 were incubated with 70 μ M of gamma-tocotrienol for 24 h. Gene expression patterns were evaluated using Sentrix HumanRef-8 Expression BeadChip from Illumina, analysed using GeneSpring GX10 software and validated using quantitative RT-PCR. A total of 100 genes were differentially expressed ($p < 0.001$) by at least 1.5 fold in response to gamma-tocotrienol treatment. Amongst the genes were IRAK3, SelS, HSPA5, HERPUD1, DNAJB9, SEPR1, C18orf55, ARF4, RINT1, NXT1, CADPS2, COG6 and GLRX5. Significant gene list was further analysed by Gene Set Enrichment Analysis (GSEA) and the Normalized Enrichment Score (NES) showed that biological processes ie. inflammation, protein transport, apoptosis and cell redox homeostasis were up-regulated in senescent fibroblasts treated with gamma-tocotrienol. These findings revealed that gamma-tocotrienol may prevent cellular aging by modulating gene expression.

RESPONSE TO TECHNICAL CHECK RESULTS

The following are my response to each of the comments given:

1) The level of English throughout this manuscript has been checked and refined. A number of grammatical errors and instances of badly worded/constructed sentences have been corrected.

2) Each bullet point provided in 'Research highlights' has been corrected and does not exceed a maximum of 85 characters including spaces.

3) The total number of tables and figures has been reduced to 6.

4) In text reference citation for 2-6 authors has been corrected throughout the manuscript.

5) Vertical lines have been deleted in all tables.

Highlights:

- Several genes were differentially expressed in response to γ -tocotrienol treatment.
- γ -Tocotrienol regulated biological processes ie. inflammation and protein transport.
- Apoptosis and cell redox homeostasis were up-regulated by γ -tocotrienol treatment.
- γ -Tocotrienol may prevent cellular aging by modulating gene expression.

1 **Gamma–tocotrienol modulated gene expression in senescent human diploid fibroblasts**
2 **as revealed by microarray analysis**

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Abstract

The effect of γ -tocotrienol, a vitamin E isomer, in modulating gene expression in cellular aging of human diploid fibroblasts (HDFs) was studied. Senescent cells at passage 30 were incubated with 70 μ M of γ -tocotrienol for 24 h. Gene expression patterns were evaluated using Sentrix HumanRef-8 Expression BeadChip from Illumina, analysed using GeneSpring GX10 software and validated using quantitative RT-PCR. A total of 100 genes were differentially expressed ($p < 0.001$) by at least 1.5 fold in response to γ -tocotrienol treatment. Amongst the genes were *IRAK3*, *SelS*, *HSPA5*, *HERPUD1*, *DNAJB9*, *SEPR1*, *C18orf55*, *ARF4*, *RINT1*, *NXT1*, *CADPS2*, *COG6* and *GLRX5*. Significant gene list was further analysed by Gene Set Enrichment Analysis (GSEA) and the Normalized Enrichment Score (NES) showed that biological processes ie. inflammation, protein transport, apoptosis and cell redox homeostasis were up-regulated in senescent fibroblasts treated with γ -tocotrienol. These findings revealed that γ -tocotrienol may prevent cellular aging by modulating gene expression.

Keywords: γ -Tocotrienol, cellular aging, human diploid fibroblasts, gene expression, microarray analysis

1. Introduction

Aging is a phenomenon associated with gradual decline in biological functions. Loss of capability to divide besides loss of cellular functions in both mitotic and post-mitotic cells is the characteristic of cellular aging (Trougakos, Saridaki, Panayotoum, & Gonos, 2006). Cellular changes that occur in the cell of an organism have direct impact on the functions of organs, systems and eventually involve the whole organism. The genetic theory of aging is proposed because of the observation that several genes affect longevity (Kirkwood, 2002). The aging process is regulated by specific genes in many organisms including yeast, *C. elegans*, fruit flies and mice. In human diploid fibroblasts, several genes including inflammatory genes, cell cycle regulatory genes, cytoskeletal genes, and metabolic genes were differentially expressed (Yoon et al., 2004) during replicative senescence and modifiable by dietary components such as antioxidants (Braam et al., 2006).

Human aging can be studied *in vitro*, specifically by using normal human diploid fibroblasts (HDFs) which undergo a limited number of cell divisions in culture and progressively reached a state of irreversible growth arrest, a process termed as replicative senescence (Trougakos et al., 2006). Senescent fibroblast cells are resistant to mitogen-induced proliferation, expressed senescence-associated β -galactosidase (SA β -gal), exhibited enlarged and flattened morphology and showed altered gene expression (Makpol, Durani, Chua, Yusof, & Ngah, 2011). Cultured human fibroblasts cells displayed age-dependent transcriptomic differences. A variety of genes involved in cell cycle regulation, immune response and inflammation, cytoskeleton, stress response and metabolism are known to be altered during cellular senescence (Zhang, Pan, & Cohen, 2003).

Approximately, about 40 micronutrients consist of vitamins, essential minerals and other compounds are required in small amount for normal metabolism and have been reported as essential components in the diet. Deficiency of macro- and micro-nutrients in aging is related to global impairments of immune functions, metabolic harmony and antioxidant defense with subsequent appearance of age-related diseases (Failla, 2003). The antioxidant vitamin E, usually alpha-tocopherol, scavenges reactive oxygen species (ROS) thus preventing oxidative damage associated with many degenerative diseases and has been suggested to act as a signaling molecule which modulates signal transduction and gene expression (Zingg et al., 2010).

Vitamin E is a lipophilic vitamin, synthesized by plants, found particularly in plant seeds and oils. There are eight naturally occurring forms of vitamin E which are α -, β -, γ -, and δ -tocopherol and α -, β -, γ -, and δ -tocotrienol (Nakamura & Omaye, 2009). Structurally, tocotrienols are different from tocopherols by the presence of three trans double-bonds in their hydrocarbon tail (Sen, Khanna, & Roy, 2007).

The prenyl side-chain of tocotrienols has been postulated to be responsible for the differential membrane distribution and metabolism of tocotrienols. α -Tocotrienol possessed a higher antioxidant activity against lipid peroxidation than α -tocopherol due to a more uniform distribution in the lipid bilayer membrane providing a more efficient interaction of the chromanol ring with lipid radicals (Kamat & Devasagayam, 1995). Tocotrienol also showed novel hypocholesterolemic activity (Qureshi, Bradlow, Salser, & Brace, 1997) in addition to more recent reports suggesting that it has neuro protective, antioxidant, anti-cancer and cholesterol lowering properties that often differ from the properties of tocopherols (Sen et al., 2007). Furthermore, tocotrienol was able to delay cellular aging by preventing

oxidative damage-induced telomere shortening in aged human fibroblast cells (Makpol, Abidin, Sairin, Mazlan, Top, & Ngah, 2010). Also tocotrienol-rich fraction (TRF) has been shown to have anti-aging properties by promoting cell cycle progression in senescent human fibroblast cells (Makpol et al., 2011). Recent research has focused on other biological functions of vitamin E that are unrelated to its antioxidant properties which include its roles in cellular signaling, gene expression, immune response, and apoptosis (Nakamura & Omaye, 2009). Therefore, this study was aimed to determine the effect of γ -tocotrienol in modulating gene expression in cellular aging using human diploid fibroblast cells.

2. Materials and Methods

2.1. Primary culture of human diploid fibroblast cells and treatment with γ -tocotrienol

This research has been approved by the Universiti Kebangsaan Malaysia Ethics Committee (Approval Project Code: FF-104-2007). Informed written consent was obtained from the parents of all subjects. Primary human dermal fibroblasts were derived from the foreskins (removed during circumcision) of three 9 to 12 year-old boys. The samples were aseptically collected and washed several times with 75 % alcohol and phosphate buffered saline (PBS) containing 1 % antibiotic–antimycotic (PAA, Austria). After removing the epidermis, the pure dermis was cut into small pieces and transferred into falcon tubes containing 0.03 % collagenase type I solution (Worthington Biochemical Corporation, USA). Pure dermis was digested in an incubator shaker at 37 °C for 6-12 h. Then, cells were rinsed with PBS before being cultured in Dulbecco Modified Eagle Medium (DMEM) containing 10 % fetal bovine serum (FBS) (PAA, Austria) and 1%

antibiotic–antimycotic at 37 °C in 5 % CO₂ humidified incubator. After 5–6 days, the cultured HDFs were harvested by trypsinization and culture-expanded in new T25 culture flasks (Nunc, Denmark) with expansion degree of 1:4. When the subcultures reached 80–90 % confluency, serial passaging was done by trypsinization and the number of population doublings (PDs) was monitored until HDFs reached senescence. For subsequent experiments, cells were used at either passage 4 (young cells, PD<12) and passage 30 (senescent cells, PD>55).

In the subsequent experiments, treated young HDFs were incubated with 50 µM γ-tocotrienol while senescent HDFs were incubated with 70 µM γ-tocotrienol for 24 h. Untreated cells were cultured in Dulbecco Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) (PAA, Austria). The media for the untreated cells was changed in parallel to the treated cells. Both untreated and treated cells were harvested on the same day (Makpol, Zainuddin, Chua, Yusof, & Ngah, 2012).

2.2. Determination of senescent biomarker SA-β-gal activity

The senescent biomarker of *in vitro* cell-aging for HDFs (SA-β-gal activity) was determined by senescent cells staining kit (Sigma, USA) according to the manufacturer's instruction. Blue staining was visible after 4 h of incubation with β-galactosidase staining solution containing 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-gal) at 37 °C. The percentage of blue cells observed in 100 cells under a light microscope was calculated.

2.3. Total RNA extraction

Total RNA from HDFs in different treatment groups was extracted using TRI Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instruction. Polyacryl Carrier (Molecular Research Center) was added in each extraction to precipitate the total RNA. Extracted total RNA pellet was then washed with 75 % ethanol and dried before being dissolved in RNase and DNase free distilled water. Total RNA was stored at -80 °C immediately after extraction. Concentration and purity of the extracted RNA was determined by Agilent 2100 bioanalyzer (Agilent Technologies, USA). RNA with RNA integrity number (RIN) ranging from 7 to 10 and absorbance ratio of A_{260} to A_{280} ranging from 1.5 to 2.1 were utilized for cDNA synthesis.

2.4. Microarray analysis

Briefly, 250 ng of total RNA from each sample was labeled by using TotalPrep RNA Amplification Kit (Ambion, Austin, USA) for cDNA synthesis. *In vitro* transcription was performed to synthesise the cRNA. Single stranded cRNA was labeled by incorporating biotin-16-UTP and cRNA was generated by incubation in the hybridization oven for 14 h at 37 °C. After 14 h incubation, 750 ng of biotin-labeled cRNA was hybridized (18 h at 58 °C) to Illumina's Sentrix HumanRef-8_v3 Expression BeadChips (Illumina, San Diego, USA). The hybridized biotinylated cRNA was detected with streptavidin-Cy3 (Amersham Biosciences, USA) and quantitated using Illumina's BeadStation S455 scanner (Illumina, San Diego, USA). Data was analysed using GeneSpring GX 10 software (Agilent Technologies, USA) and two-way analysis of variance (2-way ANOVA) was applied with the false discovery rate (FDR) for the selection of differentially expressed genes at significance level of $p < 0.001$ for baseline (data not shown) and γ -tocotrienol treated HDFs. Significant gene list

was further filtered for differences of more than 1.5 fold between γ -tocotrienol treated HDFs and untreated control. Significant genes with expression greater than 1.5 fold were selected for Gene Set Enrichment Analysis (GSEA) by using pathway studio software (Ariadne, USA) with $p < 0.05$.

2.5. Validation of microarray data using quantitative real-time RT-PCR

Quantitative real-time RT-PCR reaction was carried out to evaluate the expression of *ARF4*, *HSPA5* and *HERPUD1* using 1 μ l total RNA as template, 1 μ l of forward and reverse primers for genes of interest and iScript One-Step RT-PCR reagent with SYBR Green (Bio-Rad, USA). Primer sequences for *ARF4*, *HSPA5* and *HERPUD1* are shown below:

HSPA5 Forward: ggt gaa aga ccc ctg aca aa

Reverse: gtc agg cga ttc tgg tca tt

HERPUD1 Forward: gag cct gct ggt tct aat cg

Reverse: gaa agc tga agc cac cca ta

ARF4 Forward: ggg atg ttg gtg gtc aag at

Reverse: agc agc act gca tct ctc aa

All reactions were run in duplicate with reaction profile as follows; cDNA synthesis for 30 min at 50 °C; pre-denaturation for 2 min at 94 °C; PCR amplification for 38 cycles with 10 sec at 94 °C and 30 sec at 61 °C using Bio-Rad iCycler (Bio-Rad, USA).

3. Results

3.1. Quality control assessment of the samples

Principal Component Analysis (PCA) was used to check the quality of the microarray data. This allows viewing of separation between groups of replicates. Untreated control senescent (■) and γ -tocotrienol treated senescent HDFs (▲) were well separated and clustered into two distinct groups. Similar separation however was not observed for untreated control young (■) and γ -tocotrienol treated young HDFs (▲) (Figure 1).

3.2. Hierarchical clustering of significantly expressed genes

Cluster analysis was performed to organize genes into cluster based on their similarities of expression. Horizontal line represents a single gene and each column represents a single sample. Red color indicated the up-regulated genes, whereas green color indicated the down-regulated genes. Statistical analysis of two-way analysis of variance (2 way ANOVA) revealed a total of 100 genes were significantly regulated in tocotrienol-treated senescent HDFs compared to untreated control senescent HDFs. Clustering analysis was able to distinguish gene expression between control and tocotrienol-treated HDFs as shown by Hierarchical clustering (Figure 2).

3.3. Analysis of differentially expressed genes in γ -tocotrienol-treated HDFs

A total of 100 genes were differentially expressed in γ -tocotrienol-treated senescent HDFs as compared to untreated control senescent HDFs (Table 1). Gene Set Enrichment Analysis (GSEA) revealed the selected significant biological processes involved in response to γ -tocotrienol treatment in senescent HDFs compared to untreated control senescent HDFs (Table 2). Positive value of Normalized Enrichment Score (NES) indicated the up-regulated process. The biological processes that were modulated by γ -tocotrienol treatment included

the negative regulation of tumor necrosis factor production (inflammation), negative regulation of interleukin-6 production (inflammation), negative regulation of caspases activity (apoptosis), response to stress, transport protein and cell redox homeostasis. Pathway analysis was carried out for selected genes (*ARF4*, *HSPA5* and *HERPUD1*) (Figure 3) and validation on the microarray data was carried out by real time RT-PCR. Results showed an up-regulation of the selected genes in γ -tocotrienol-treated senescent HDFs as compared to untreated control senescent HDFs (Figure 4). Comparison between microarray and RT-PCR data showed that there was a consistent expression pattern of *ARF4*, *HSPA5* and *HERPUD1* genes which are involved in protein transport and negative regulation of apoptosis.

4. Discussion

Non-antioxidant activities of vitamin E using α -tocopherol has been increasingly reported. For instance, α -tocopherol and α -tocopheryl phosphate were reported to be involved as mediators of lipid metabolism by modulating signal transduction and gene expression (Zingg et al., 2010). Our findings from the present study showed that the another form of vitamin E, γ -tocotrienol was able to modulate gene expression in human diploid fibroblasts. In response to γ -tocotrienol treatment, a total of 100 genes were differentially expressed in senescent HDFs which included *IRAK3*, *SelS*, *HSPA5*, *HERPUD1*, *DNAJB9*, *SEPR1*, *C18orf55*, *ARF4*, *RINT1*, *NXT1*, *CADPS2*, *COG6* and *GLRX5*. Gene Set Enrichment Analysis (GSEA) revealed that *IRAK3* was involved in inflammation process specifically in the negative regulation of tumor necrosis factor production and negative regulation of interleukin-6 production. *IRAK3* encodes for one of the interleukin receptor-associated kinase (IRAK) family and has a role in both positive and negative regulation of signal transduction.

This gene is also known as *IRAK-M* and previously, the expression of human *IRAK-M* was limited to monocytes and macrophages (Flannery & Bowie, 2010). However in the present study, its expression was significantly increased in γ -tocotrienol-treated senescent fibroblast cells.

The aging process is attributed to the presence of low chronic inflammation resulting in a stressed condition. Genes related to inflammation are relevant after taking into account that the innate immunity is more involved during inflammation. A chronic inflammatory status called inflame-aging appears to be the major component of common age-related diseases including cardiovascular diseases and infections (Franceschi et al., 2000). Among the inflammatory agents that have been identified were interleukin-6, interleukin-1 β , cyclooxygenase 2 and tumor necrosis factor (TNF). Up-regulation of pro-inflammatory mediators was observed during aging due to an age-related redox imbalance that activates several pro-inflammatory signaling pathways. Dysregulation of these cytokines promotes inflammation and tissue damage. This indicated that aging is accompanied by chronic low-grade inflammation state showed by 2 to 4 fold increase in serum levels of inflammatory mediators such as C-reactive protein, tumor necrosis factor (TNF) and cyclooxygenase 2 (COX2) (Chung et al., 2009). Up-regulation of *IRAK3* in this study may indicate protection against cellular aging and age-related diseases.

In the present study we also found that *selenoprotein S* (*SelS*) was significantly up regulated in γ -tocotrienol-treated senescent fibroblast cells. Selenoprotein S is involved in negative regulation of tumor necrosis factor production, negative regulation of interleukin-6 production and cell redox homeostasis. It encodes for an endoplasmic reticulum (ER) transmembrane protein and is present in a variety of tissues such as liver, skeletal muscle and

adipose tissue (Du, Liu, & Huang, 2010). Selenoproteins contain the twenty-first amino acid, selenocysteine, reported to be involved in cellular defenses against oxidative damage. Furthermore, these proteins are involved in important metabolic and developmental pathways in response to environmental challenges (Stoytcheva & Berry, 2009). Many of the selenoproteins are involved in protection against oxidative stress or in maintaining cellular redox balance. SelS is considered as an important component of retro-translocation channel in endoplasmic reticulum-associated protein degradation (ERAD). This ER membrane protein functions in stress responses to prevent the deleterious consequences of accumulation of misfolded proteins which has been linked to immune and inflammatory processes. Previous findings have suggested that SelS may regulate cytokine production in macrophages, and a regulatory loop between cytokines and SelS has been proposed to play a key role in controlling the inflammatory response (Gao et al., 2006). Negative regulation of TNF and interleukin 6 production by *IRAK3* and *SelS* in γ -tocotrienol-treated senescent HDFs observed in this study may indicate inhibition of chronic inflammatory processes that normally accompanies cellular aging. Thus this may suggest one of the mechanisms involved for γ -tocotrienol in slowing down cellular aging of HDFs.

γ -Tocotrienol also modulated the expression of genes that are involved in protein transport. Our results showed that ADP-ribosylation factor 4 (*ARF4*) was significantly up-regulated in γ -tocotrienol treated senescent HDFs. ADP-ribosylation factors have been implicated in several important cellular processes, including membrane trafficking and activation of phospholipase D (Kim, Hayashi, Lo, Yang, Yoo, & Lee, 2003). They are members of the Ras super family of small guanine nucleotide-binding proteins, and were initially identified as proteins that stimulate the ADP-ribosyl transferase activity of cholera

toxin *in vitro*. ARFs are ubiquitously expressed from yeast to mammals and function primarily in the regulation of membrane trafficking. Based on their deduced amino acid sequence, protein size, and phylogenetic analysis, these proteins can be divided into three groups which are class I (ARF1, ARF2, ARF3), class II (ARF4, ARF5), and class III (ARF6). Previous report has demonstrated that over expression of ARF4 in U373MG cells suppresses N-(4-hydroxyphenyl) retinamide (4-HPR)-induced cell death. The findings from yeast-based functional screening in *S. cerevisiae* which facilitates the identification of anti-apoptotic mammalian genes showed that ARF4 may function as a novel suppressor of apoptosis (Woo et al., 2009).

Heat shock proteins (Hsps) have been studied for many years and there are evidence that demonstrated the role of Hsp up-regulation in tissues and cell protection in a wide variety of stress conditions. Heat shock proteins (HSPs) belong to a class of highly conserved proteins that act physiologically as molecular chaperones to stabilize existing proteins against aggregation and mediate the folding of newly translated proteins in the cytosol and other organelles (Lee et al., 2009). Additionally, they have been shown to demonstrate anti-apoptotic effects against a wide range of both physical and chemical apoptotic stimuli (Garrido, Gurbuxan, Ravagnan, & Kroemer, 2001). Similar finding was found in our study, whereby the *HSPA5* (heat shock 70 kDa protein 5) also known as BiP or GRP78 (glucose-regulated protein, 78 kDa) which is involved in negative regulation of caspase activity in response to stress was significantly increased in γ -tocotrienol-treated senescent HDFs. Besides, treatment with γ -tocotrienol in senescent HDFs caused up-regulation of *HERPUD1* which is also involved in negative regulation of caspase activity in response to stress. HERP (homocysteine-induced ER protein) which was recently identified and characterized as an ER

membrane protein was up-regulated in response to ER stress. Induction of HERP was reported to be involved in the protection of cells against ER stress. HERP stabilizes ER Ca^{2+} homeostasis and mitochondrial functions in neuronal cells during ER stress (Chan et al., 2004). Thus the findings from the present study showed that treatment with γ -tocotrienol in senescent HDFs may delay cellular aging of HDFs by modulating cellular stress responses and regulating the apoptosis pathway.

Oxidative stress is known to be involved in a number of pathological conditions, including neurodegeneration, cardiovascular disease and stroke, and even plays a role in natural aging. Oxidative stress occurs when the levels of oxidants are higher than the levels of antioxidants, thus overwhelming the system. The imbalance between prooxidants and antioxidants leads to an accumulation of oxidative damage with age in a variety of macromolecules resulting in a progressive loss of functional cellular processes, leading to the aging phenotype (Ott, Gogvadze, Orrenius, & Zhivotovsky, 2007). A progressive rise of oxidative stress due to the altered redox homeostasis appears to be one of the hallmarks of the aging process. Changes in the pattern of gene expression through ROS-sensitive transcription factors give rise to both aging and inflammation phenotypes. Chronic oxidative stress and inflammatory reactions lead to many age-associated diseases such as atherosclerosis and arthritis (Lavrovsky, Chatterjee, Clark, & Roy, 2000).

Cells have developed both non-enzymatic and enzymatic defense mechanisms to counteract the deleterious effects of oxidative stress by either detoxifying reactive oxygen species (ROS) or repairing ROS induced damage. Non-enzymatic examples of antioxidant include vitamin C, vitamin E, ubiquinone, flavonoids and glutathione (GSH) and examples of enzymatic scavengers include catalase, glutathione peroxidase, thioredoxin, Cu/Zn

superoxide dismutase (Cu/Zn SOD), Mn/superoxide dismutase (MnSOD), and glutaredoxins (Martindale & Holbrook, 2002). Interestingly, our finding showed that γ -tocotrienol intervention in senescent HDFs caused increased expression of glutaredoxin 5 homolog (*GLRX5*) gene involved in cell redox homeostasis. Glutaredoxins are glutathione dependent oxidoreductases that help maintain cellular redox homeostasis in the cell. The glutaredoxin system consists of GSH, NADPH, and GSH reductase. The mammalian system contains three known members of the glutaredoxin family Grx1, Grx2, and Grx5. The recently discovered glutaredoxin 5, a monothiol glutaredoxin, is hypothetically localized to the mitochondria. The loss of Grx5 in yeast leads to constitutive oxidative damage, sensitization to ROS, iron accumulation, and inactivation of iron–sulfur (Fe–S) cluster containing enzymes (Rodríguez-Manzaneque, Tamarit, Bellí, Ros, & Herrero, 2002). Our findings are in agreement with a recent study that showed over expression of glutaredoxin 5 caused decreased in DNA fragmentation and protect cells against H₂O₂ induced apoptosis and ROS formation (Linares, Xing, Govoni, Chen, & Mohan, 2009).

In summary, the findings from this study elucidated the non-antioxidant properties of a vitamin E isomer, γ -tocotrienol in delaying cellular aging indicated by regulation of protective biological processes through the modulation of gene expression in human diploid fibroblasts.

5. Conclusion

γ -Tocotrienol may delay or protect against cellular aging of human diploid fibroblasts by modulating genes expression that are involved in biological processes related to oxidative stress such as inflammation, protein transport, apoptosis and cell redox homeostasis.

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342 **Acknowledgements**

343 This study was funded by the Ministry of Higher Learning under the Fundamental Research
344 Grant Scheme UKM-FF-03-FRGS0043-2009 and Universiti Kebangsaan Malaysia grant FF-
345 328-2009.

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Figure legends:

Fig. 1. Principal Component Analysis (PCA) plot was derived from biological replicates of HDFs (n = 6). Two dimensional PCA plot showing clustering of the different treatment groups; untreated control senescent HDFs (red ■), γ -tocotrienol treated senescent HDFs (red ▲), untreated control young HDFs (blue ■) and γ -tocotrienol treated young HDFs (blue ▲).

Fig. 2. Hierarchical cluster analysis of genes shows the differential expression of genes in young and senescent HDFs in response to γ -tocotrienol treatment ($p < 0.001$). Samples were clustered under their conditions.

Fig. 3. Network of common target for the molecular relationship of selected differentially expressed genes in γ -tocotrienol treated senescent HDFs. The highlighted genes were the regulator for the downstream genes.

Fig. 4. Three genes were selected for validation by one step real time RT-PCR technique. All data were normalized to the housekeeping gene, *GAPDH*. The expression patterns obtained through real time RT-PCR were consistent with the microarray results. *ARF4* (A), *HSPA5* (B) and *HERPUD1* (C) were significantly up-regulated in γ -tocotrienol treated senescent HDFs compared to untreated control ($p < 0.05$).

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459

460 Table 1. Genes that are differentially expressed in γ -tocotrienol-treated senescent HDFs

Accession	Symbol	Expression	Fold change
NM_203453.2	PPAPDC2	down-regulated	1.82187
NM_005768.5	OACT5	down-regulated	2.454743
NM_002053.1	GBP1	down-regulated	3.722773
NM_032549.1	IMMP2L	down-regulated	1.596754
NM_012134.1	LMOD1	down-regulated	1.924233
NM_000725.2	CACNB3	down-regulated	2.057523
NM_018530.1	GSDML	up-regulated	2.411995
NM_022055.1	KCNK12	up-regulated	2.671492
NM_181463.1	MRPL55	up-regulated	3.117942
NM_001005914.1	SEMA3B	up-regulated	1.764685
NM_020751.1	COG6	up-regulated	2.937211
NM_002245.2	KCNK1	up-regulated	4.019881
NM_018142.1	C8orf35	up-regulated	2.102989
NM_001017370.1	LOC387921	up-regulated	1.93137
NM_203301.1	FBXO33	up-regulated	1.556359
NM_017758.2	ALKBH5	up-regulated	3.526197
NM_015984.1	UCHL5	up-regulated	2.233388
NM_023077.1	C1orf163	up-regulated	1.564331
NM_021934.3	C12orf44	up-regulated	2.943061
NM_017986.2	GPR172B	up-regulated	2.2754
NM_025057.1	C14orf45	up-regulated	4.049992
NM_145045.3	MGC20983	up-regulated	1.766083
NM_133443.1	GPT2	up-regulated	6.051548
NM_031479.3	INHBE	up-regulated	24.43676
NM_001010990.1	HERPUD1	up-regulated	6.54086
NM_022374.1	ARL6IP2	up-regulated	2.43751
NM_022771.3	TBC1D15	up-regulated	2.361947

NM_003136.2	SRP54	up-regulated	2.621556
NM_002703.3	PPAT	up-regulated	1.981983
NM_013242.2	C16orf80	up-regulated	1.569004
NM_206825.1	GNL3	up-regulated	4.279188
NM_145805.1	ISL2	up-regulated	2.056091
NM_001012398.1	AKTIP	up-regulated	2.62478
NM_001080392.1	KIAA1147	up-regulated	3.374165
NM_014305.1	TGDS	up-regulated	2.466504
NM_017443.3	POLE3	up-regulated	3.112311
NM_012463.2	ATP6V0A2	up-regulated	2.743501
NM_024596.1	MCPH1	up-regulated	1.539281
NM_022366.1	TFB2M	up-regulated	2.297854
NM_016115.3	ASB3	up-regulated	2.506017
NM_001080485.1	ZNF275	up-regulated	1.891536
NM_017954.8	CADPS2	up-regulated	2.474965
NM_016433.3	GLTP	up-regulated	1.592448
NM_003408.1	ZFP37	up-regulated	2.532156
NM_133173.2	APBB3	up-regulated	2.074302
NM_021800.2	DNAJC12	up-regulated	4.182389
NM_173622.2	NBLA10383	up-regulated	3.172007
NM_018456.4	EAF2	up-regulated	7.062471
NM_004569.2	PIGH	up-regulated	2.691048
NM_014445.2	SERP1	up-regulated	3.263752
NM_000785.3	CYP27B1	up-regulated	7.801915
NM_032758.3	PHF5A	up-regulated	2.000297
NM_024818.2	UBE1DC1	up-regulated	2.508222
NM_181291.1	WDR20	up-regulated	1.900369
NM_032331.2	MGC2408	up-regulated	2.694464
NM_024310.2	PLEKHF1	up-regulated	2.931577
NM_020705.1	TBC1D24	up-regulated	3.026798
NM_203472.1	SELS	up-regulated	2.317949

NM_024887.2	DHDDS	up-regulated	3.077372
NM_017748.3	CCDC49	up-regulated	1.65632
NM_006207.1	PDGFRL	up-regulated	3.129943
NM_001407.1	CELSR3	up-regulated	3.975146
NM_016948.1	PAR6A	up-regulated	2.234757
NM_005927.3	MFAP3	up-regulated	1.936899
NM_002162.2	ICAM3	up-regulated	3.910169
NM_018145.1	FAM82C	up-regulated	1.63418
NM_025147.2	COQ10B	up-regulated	1.772477
NM_007199.1	IRAK3	up-regulated	2.630288
NM_005088.2	CXYorf3	up-regulated	2.224705
NM_014171.3	CRIP1	up-regulated	2.496879
NM_004794.2	RAB33A	up-regulated	4.259968
NM_198077.1	C1orf52	up-regulated	1.971783
NM_012328.1	DNAJB9	up-regulated	7.301712
NM_001660.2	ARF4	up-regulated	2.159794
NM_001660.2	ARF4	up-regulated	3.664654
NM_018130.2	SHQ1	up-regulated	1.824473
NM_021930.3	RINT-1	up-regulated	1.687643
NM_004634.2	BRPF1	up-regulated	1.851133
NM_138381.1	MGC15763	up-regulated	3.638718
NM_007022.3	CYB561D2	up-regulated	3.542795
NM_130781.1	RAB24	up-regulated	2.674383
NM_021237.3	SELK	up-regulated	3.304395
NM_005175.2	ATP5G1	up-regulated	2.527163
NM_014177.1	C18orf55	up-regulated	1.589782
NM_032102.1	SRP46	up-regulated	2.544408
NM_175856.4	CSS3	up-regulated	1.73414
NM_006545.4	TUSC4	up-regulated	5.893672
NM_016417.2	GLRX5	up-regulated	1.718225
NM_001080403.1	FLJ22184	up-regulated	3.315912

NM_015394.4	ZNF10	up-regulated	2.517444
NM_005347.2	HSPA5	up-regulated	5.951358
NM_005444.1	RQCD1	up-regulated	2.066589
XM_940962.1	LOC124512	up-regulated	1.787957
NM_001031677.2	RAB24	up-regulated	3.279507
NM_001031677.2	RAB24	up-regulated	2.376335
NM_001015882.1	bA16L21.2.1	up-regulated	2.021612
NM_153213.2	ARHGEF19	up-regulated	2.899712
NM_013248.2	NXT1	up-regulated	3.503187
NM_201262.1	DNAJC12	up-regulated	4.301361
NM_201262.1	DNAJC12	up-regulated	2.60083

461

462 Table 2. Selected significant biological processes that were modulated in senescent HDFs after γ -tocotrienol treatment for 24 h
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<i>Biological process</i>	<i>Gene Set Enrichment Analysis (GSEA)</i>		
	Normalized Enrichment Score	Gene symbol	Description
Negative regulation of tumor necrosis factor production	1.54164		
		IRAK3	Interleukin-1 receptor-associated kinase 3
		Sels	selenoprotein S
Negative regulation of interleukin-6 production	1.65684		
		IRAK3	Interleukin-1 receptor-associated kinase 3
		Sels	selenoprotein S
Negative regulation of caspase activity	1.40798		
		HSPA5	Heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)
		HERPUD1	Homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1
Response to stress	1.61171		
		HSPA5	Heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)
		HERPUD1	Homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1
		DNAJB9	DnaJ (Hsp40) homolog, subfamily B, member 9
		SERP1	Stress-associated endoplasmic reticulum protein 1
Protein transport	1.68316		

Cell redox homeostasis	1.56752	C18orf55	Chromosome 18 open reading frame 55
		ARF4	ADP-ribosylation factor 4
		RINT1	RAD50 interactor 1
		SERP1	Stress-associated endoplasmic reticulum protein 1
		NXT1	NTF2-like export factor 1
		CADPS2	Ca ²⁺ -dependent activator protein for secretion 2
		COG6	Component of oligomeric golgi complex 6
		GLRX5	Glutaredoxin 5 homolog
		Sels	Selenoprotein S

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Figure 1

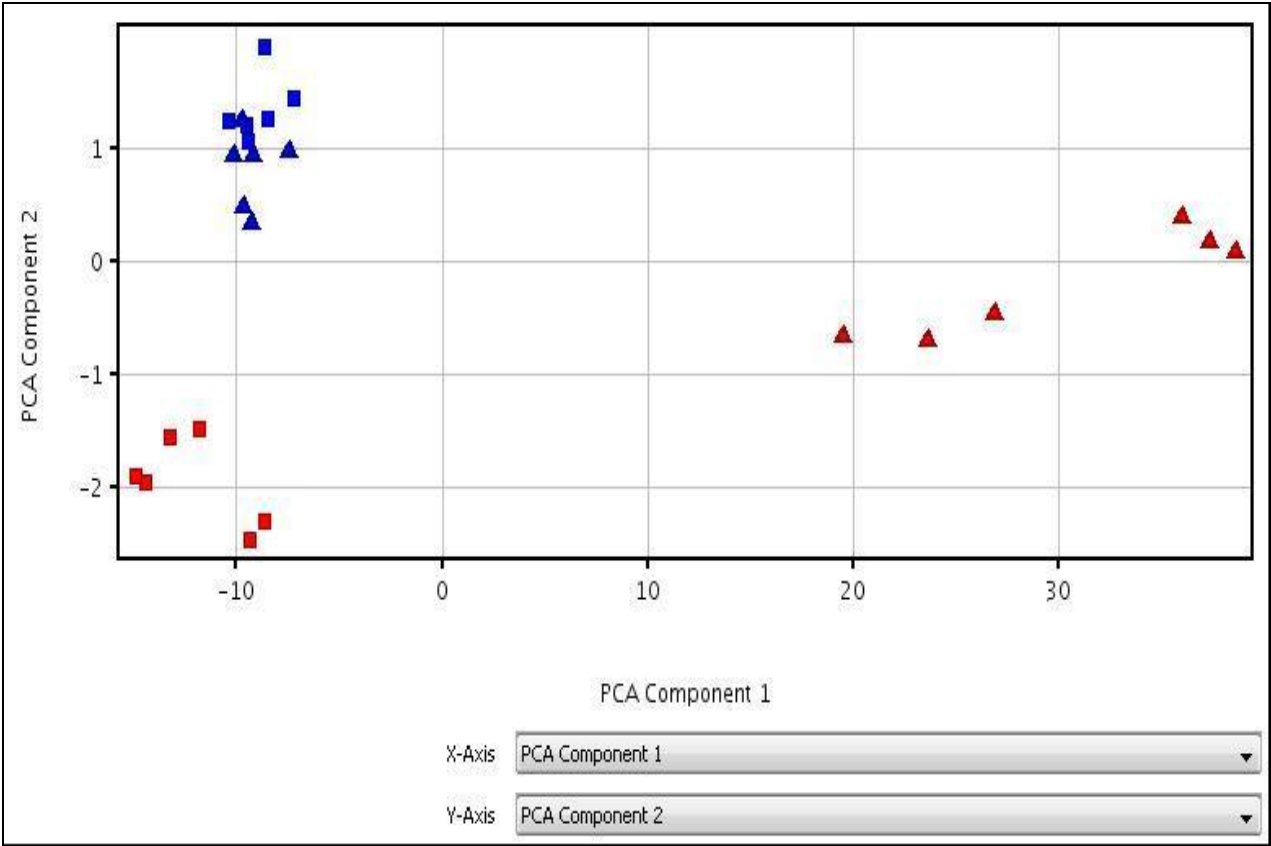


Figure 2

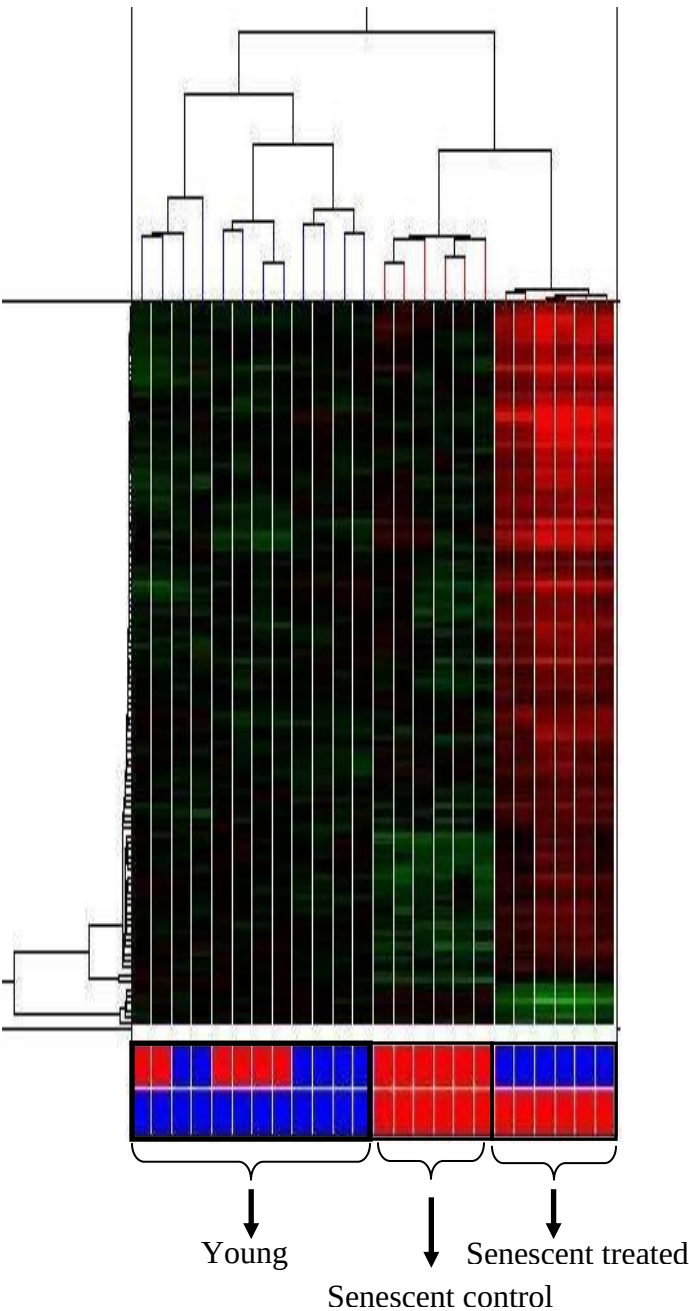


Figure 3

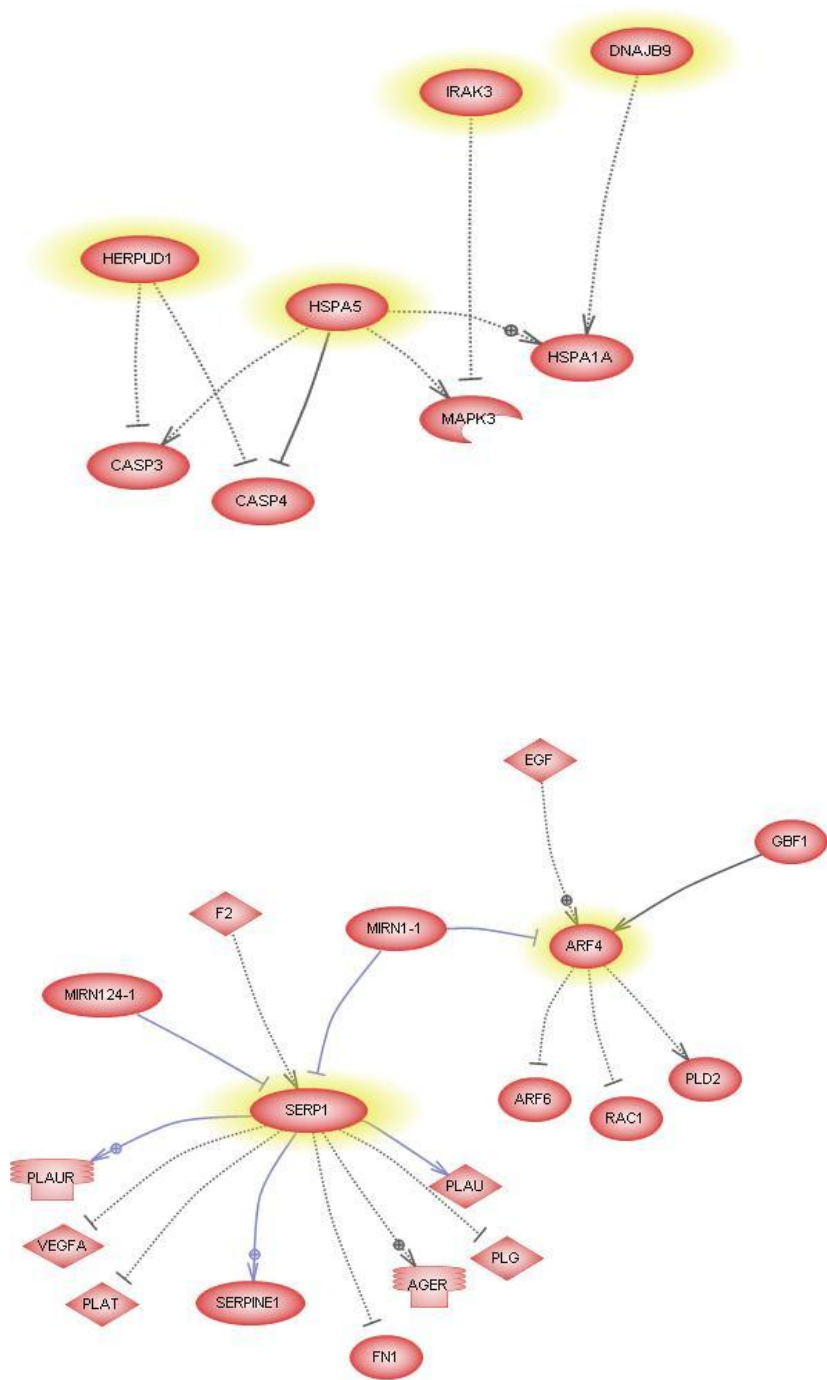


Figure 4

