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Research article

Wheat germ oil vitamin E cytoprotective effect and its nutrigenomics signature in human hepatocyte lipotoxicity

Desirée Bartolini [a], Rita Marinelli [a], Anna Maria Stabile [b], Tiziana Frammartino [c,d], Angela Guerrini [c,d], Stefano Garetto [c,d], Jacopo Lucci [c,d], Anna Migni [a], Linda Zatini [a], Giada Marcantonini [a], Mario Rende [b], Francesco Galli [a,*]

[a] Department of Pharmaceutical Sciences, Lipidomics and Micronutrient Vitamins Lab. and Human Anatomy Lab., University of Perugia, 06126 Perugia, Italy
[b] Department of Medicine and Surgery, Section of Human, Clinical and Forensic Anatomy, University of Perugia, Perugia, Italy
[c] Natural Bio-Medicine SpA, Loc. Aboca 20, 52037 Sansepolcro, AR, Italy
[d] Innovation and Medical Science Division, Aboca SpA Societa Agricola, Loc. Aboca 20, 52037 Sansepolcro, AR, Italy

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ABSTRACT

Wheat germ oil (WGO) is rich in α-tocopherol (vitamin E, VE), a vitamin that has long been suggested to exert hepatoprotective effects. In this study, this function of WGO-VE and its transcriptomics fingerprint were investigated in comparison with RRR−α-tocopherol and *all-rac*-α-tocopherol (nVE and sVE, respectively), in human liver cells treated with oleic acid (OA) to develop steatosis and lipotoxicity. Used in chemoprevention mode, all the VE formulations afforded significant reduction of the OA-induced steatosis and its consequent impact on lipotoxicity indicators, including ROS production and efflux (as H_2O_2), and apoptotic and necrotic cell death. A trend toward a better control of lipotoxicity was observed for WGO-VE and nVE compared to sVE.

Gene microarray data demonstrated that these effects of VE formulations were associated with significantly different responses of the cellular transcriptome to compensate for the modifications of OA treatment, including the downregulation of cellular homeostasis genes and the induction of genes associated with defects of liver cell metabolism, fibrosis and inflammation, liver disease and cancer. Ingenuity Pathway Analysis data showed that WGO-VE modulated genes associated with liver carcinogenesis and steatosis, whereas nVE modulated genes involved in liver cell metabolism and viability biofunctions; sVE did not significantly modulate any gene dataset relevant to such biofunctions.

In conclusion, WGO-VE prevents lipotoxicity in human liver cells modulating genes that differ from those affected by the natural or synthetic forms of pure VE. These differences can be captured by precision nutrition tools, reflecting the molecular complexity of this VE-rich extract and its potential in preventing specific cues of hepatocellular lipotoxicity.

1. Introduction

Wheat germ (WG) is one of the richest sources of α-tocopherol (α-TOH) in the plant kingdom, and one of the first food ingredients used right one century ago in the pioneering studies that led to identify vitamin E (VE) as an essential nutrient important in animal fertility (H. M. Evans and Bishop, 1922). WG was the experimental material in which the stability of VE was first associated with processes of lipid autoxidation and with its "antioxygenic" properties (Cummings and Mattill, 1931; H. M. Evans and Burr, 1925; H.M. Evans, Emerson and Emerson, 1936; Mattill et al., 1924).

Nowadays, VE is widely utilized in industrial processes as fat-soluble antioxidant and lipid stabilizer of food, cosmetic, pharmaceutical and nutrition products. Synthetic VE (sVE) is the most common form of VE utilized for these applications, with worldwide production and commercialization figures expected to growth over 70 K-tons/year by 2026 (Ahuja and Rawat, 2020). sVE consists of a racemic mixture (*all-rac*) of the S and R stereoisomers of α-TOH molecule, thus presenting all the possible stereoisomeric combinations at the 3 chiral centers in the side chain (Suppl. Figure S1). Moreover, this form is usually esterified to an acetate molecule or to other organic acids at the hydroxyl group in

* Corresponding author.
E-mail address: francesco.galli@unipg.it (F. Galli).

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position 6 of the chroman ring to optimize compound stability and organoleptic properties.

A small fraction of VE is produced by extraction from plant raw materials and commercialized under its natural form (α-TOH with RRR configuration in the 3 chiral centers; Suppl. Figure S1, left upper structure), but the estimates indicate important growths of demand for this form in the next years by the fact that consumers are progressively opting for chemical-free and naturally sourced products (Ahuja and Rawat, 2020).

Natural VE (nVE) is commonly obtained as an oily product in pure form or as main component of lipid mixtures by the extraction of plant raw materials, including green leafy vegetables, nuts and seeds, with most common sources including soybean, sunflower and wheat germ (recently reviewed in (P. Torquato, Marinelli et al., 2020)).

Wheat germ oil (WGO) contains both tocopherols and tocotrienols, but α-TOH is by far the most abundant vitamer. Its relative concentrations are the highest amongst edible oils ranging from 100 to 300 mg/100 g of oil (w/w) (Eisenmenger and Dunford, 2008; H.M. Evans et al., 1936; Ghafoor et al., 2017).

The health-promoting properties of WGO include an increased antioxidant protection of tissues (Leenhardt et al., 2008), which has primarily been associated with WGO-VE antioxidant function. Other lipid factors in WGO may play a role in this context, such as polycosanols, carotenoids, phytosterols and essential fatty acids, which have been reported to provide anti-inflammatory and lipid lowering effects [recently reviewed in (Ghafoor et al., 2017; Kumar and Krishna, 2015)].

Besides a role as fat-soluble antioxidant, VE has been demonstrated to influence gene expression in several tissues [recently reviewed in

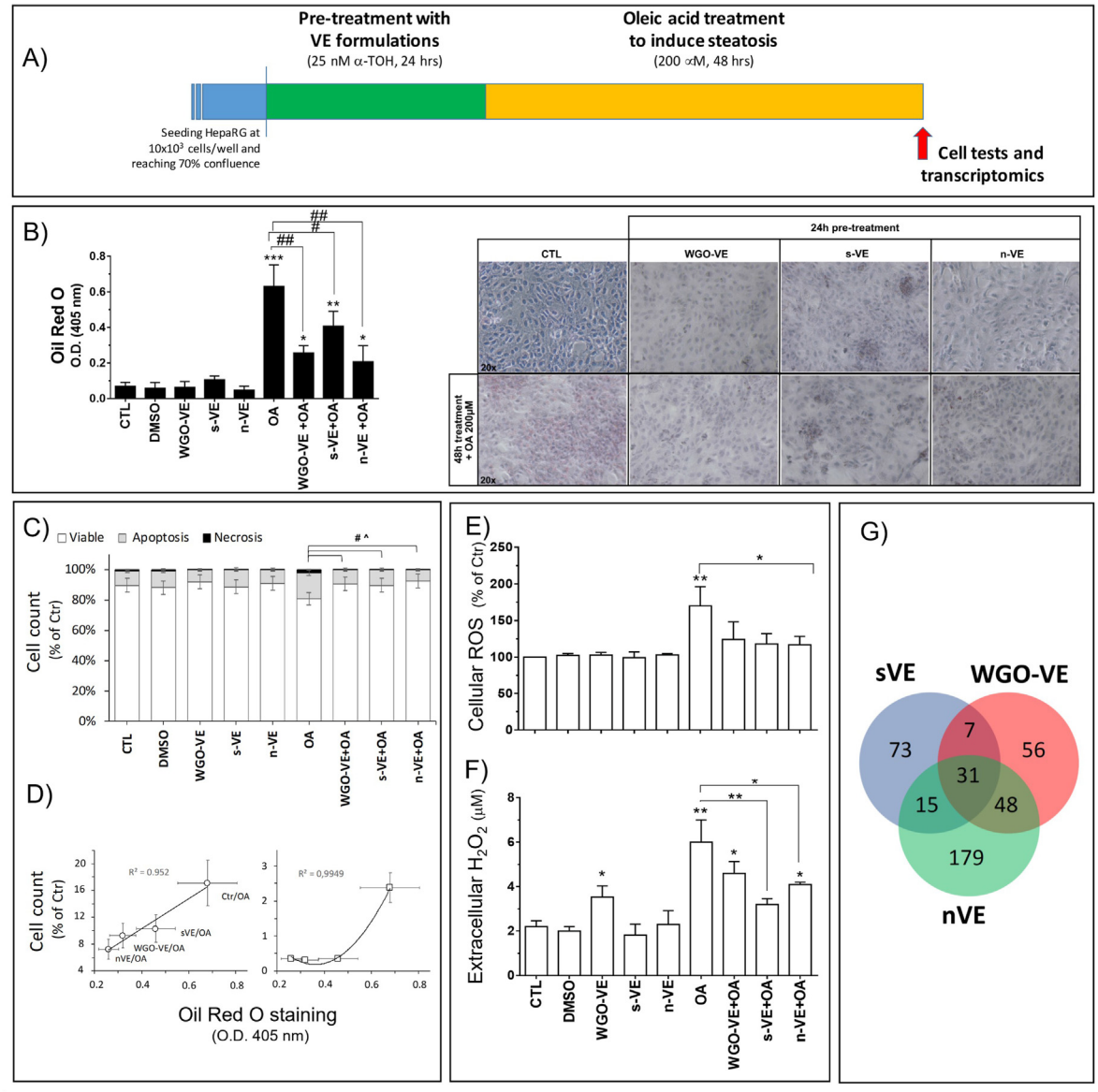


Figure 1. Effects of vitamin E formulations on the OA-induced steatosis and lipotoxicity of HepaRG, and corresponding transcriptional response. (A) Treatment protocol; this included a 24 h pretreatment phase with the three forms of VE, namely nVE (RRR-α-Tocopherol), sVE (all-rac-α-Tocopherol), and WGO-VE (containing 0.2% w/w total vitamin E, average relative composition: 60 % minimum in α-tocopherol, 25–30% γ + β−tocopherol, 5–8% γ-tocotrienol, other forms <1%), all administered to the final concentration of 25 nM α-Tocopherol in the cell culture medium, and a 48 h treatment phase with 200 μM OA to induce steatosis. (B) ORO staining of cellular triglyceride (left chart) and light microscopy identification of their subcellular localization in LD (right pictures); (C) cell death analysis by FACS scan analysis, and AV and PI staining; (D) correlation between apoptotic or necrotic cell death and levels of cellular lipids as determined by ORO staining (left and right panels, respectively); (E) cellular ROS and (F) efflux of H_2O_2; (G) Venn Diagram of the transcriptional response to VE formulations. t-test: *p < 0.05; **p < 0.001; ***p < 0.0001 (all data); #p < 0.05 (apoptosis data), ^ p < 0.05 (necrosis data).

(Rimbach et al., 2010; P. Torquato, Marinelli et al., 2020; Zingg, 2019)], including the liver (Podszun et al., 2020; Rimbach et al., 2010; Valastyan et al., 2008). Both the antioxidant and gene modulation properties of VE have been indicated to play an important role in protecting the liver from a series of threats, including free radical-generating agents as CCl_4 (Parola et al., 1992), defects of anti-peroxidative defenses (Carlson et al., 2016), and lipotoxicity that is the underlaying event of non-alcoholic fatty liver disease (NAFLD) (reviewed elsewhere in (Perumpail et al., 2018; Podszun et al., 2020; Sato et al., 2015; Svegliati-Baroni et al., 2019)).

To our knowledge, such promising effects of VE have not been investigated before in the case of WGO-VE. Therefore, in the present study, we explore the WGO-VE hepatoprotective function in human liver cells treated with oleic acid (OA) to develop steatosis and lipotoxicity, and the transcriptional response associated with this function was also evaluated using a microarray technique. The liver cell protection and transcriptional effects of WGO-VE were studied in comparison with nVE and sVE, in an attempt to discern the role of the vitamin from that of other components present in the WGO phytocomplex.

2. Materials and methods

2.1. Cell line

HepaRG human progenitor hepatic cells (Thermo Fisher Scientific) were seeded at a density of 10,000 cells/well and maintained at 37 °C in a humidified 5% CO_2 atmosphere in William's *E medium* (Thermo Fisher Scientific) supplemented with 1% Glutamax (Gibco, Thermo Fisher Scientific; catalog number: 35050061), 5 μg/mL human insulin (Lonza) and 50 μM hydrocortisone hemisuccinate (Sigma-Aldrich). Cells were used between passage 2 and 10, and passages were implemented using trypsin-EDTA (Euroclone).

2.2. Cell treatments

Cell treatments were as described in Figure 1A and included a pretreatment phase with VE formulations and a treatment phase with OA to induce lipotoxicity.

2.2.1. Vitamin E pretreatments

HepaRG cells were used at 70% confluence for the pretreatment (24 h) with vehicle (DMSO, Sigma-Aldrich; final concentration in the cell culture medium of 0.001% vol/vol) or VE in six-well plates. The three forms investigated in this study were:

- nVE (RRR-α-TOH, product number: T3634, Sigma-Aldrich),
- sVE (*all-rac*-α-Tocopherol, product number: 5.00949, SAFC, Merck KGaA, Darmstadt, Germany),
- WGO-VE, (*Triticum aestivum L.* germ oil, Aboca S.p.A, Sansepolcro, Italy), containing 0.25 % w/w total vitamin E (average relative composition: min. 60 % in α-tocopherol, 25–30 % γ+β−tocopherol, 5–8 % γ-tocotrienol, other forms <1%).

Pretreatments with the three forms of VE were preliminarily investigated for possible toxicity effects (assessed by cell viability and apoptosis tests) and induction of steatosis; the latter was a significant effect of the pretreatment with WGO (not shown). This unwanted effect was prevented using a quantity of WGO corresponding to a final concentration of α-TOH in the culture medium of 25 nM. Therefore, the same concentration was applied to the cell tests performed with other forms of VE used for comparison with WGO-VE.

2.2.2. Treatment with oleic acid to induce steatosis

Steatosis and lipotoxicity were induced in HepaRG cells using free fatty acid (FFA) supplementation protocol according to (Bartolini et al., 2017; Ricchi et al., 2009), with some modifications. In brief, after pretreatment with VE formulations, oleic acid (OA, BioReagent from Sigma-Aldrich, product number: O1383) was utilized as FFA to induce steatosis in HepaRG cells. Stock solutions of OA were prepared in ethanol and were further diluted in William E complete medium containing 10% FBS to the final concentration of 200 μM in the culture medium for cell supplementation. Cell treatments with this FFA lasted for 48 h.

Controls experiments included: a) untreated cells (tests labeled as CTR), b) pretreated cells not exposed to OA during the treatment phase and c) cells pretreated with the vehicle of VE forms that was a control for the effect of VE pretreatments (as main endpoint of the study).

2.3. Cell viability and apoptosis

Cell viability was assessed by MTT test (Sigma-Aldrich) according with (Bartolini et al., 2015). Briefly, after pre-treatment with VE formulations and treatment with OA, the cells were incubated for 3 h at 37 °C with fresh medium containing the MTT solution; then the formazan crystals were dissolved in MTT solubilization solution (10% Triton X-100 plus 0.1 N HCl in anhydrous isopropanol) and the absorbance was recorded at 570 nm using a microplate reader (DTX880, Multimode Detector Beckman Coulter). Other details and calculation of cell viability data were as in (Bartolini et al., 2015).

Apoptosis was investigated in HepaRG cells (2.5×10^5 cells/well) by cytofluorimetric analysis using the fluorescent probes propidium iodide (PI, Clontech) and Annexin V, Alexa Fluor™ 488 conjugate (Thermo Fisher Scientific) according to (Knani et al., 2019).

2.3.1. Cellular reactive oxygen species (ROS)

Intracellular ROS were measured by the oxidative conversion of the intracellular probe 2′,7′-dichlorofluorescein-diacetate (DCFH-DA; Sigma-Aldrich) to the fluorescent derivative 2′,7′-dichlorofluorescein (DCF), while extracellular ROS were determined as H_2O_2 with a microplate assay procedure utilizing the Amplex™ Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen) as described in (Volpi et al., 2021).

2.4. Oil Red O staining of cellular lipids

Cellular lipids were measured by Oil Red O (ORO) staining according to the procedure described in (Bartolini et al., 2017). Briefly, after the treatments, the HepaRG cells were fixed in 4% paraformaldehyde (PFA, Leica) for 15 min at room temperature, and then washed three times with phosphate-buffered saline (PBS, Sigma-Aldrich) before staining for 5 min with 0.5% ORO solution (Sigma-Aldrich) and then for 1 min with hematoxylin solution (Sigma-Aldrich). After rinsing with distilled water, slides were assessed for qualitative and semiquantitative analysis by optical microscopy (Olympus CK2 Inverted Binocular Microscope for Phase Tissue Culture) with photographic camera (Olympus DP50). To quantify the cellular content of ORO, the cell pellet was incubated for 10 min with 100 μl of isopropanol and the absorbance of the extract was assessed at 510 nm using a DTX880, Multimode Detector Beckman Coulter reader.

2.4.1. Gene expression profile

Whole transcriptome expression profile was evaluated using a Human Clariom™ GO Screen Assay Applied Biosystems, Thermo Fisher Scientific) performed on a GeneTitan MC Instrument Applied Biosystems, Thermo Fisher Scientific). Total RNA was extracted from cells lysates using a QIAsymphony RNA Kit (Qiagen,) with the QIAsymphony SP instrument (Qiagen). The quality and quantity of RNA were determined with a Nanodrop2000 spectrophotometer (Thermo Fisher Scientific) at 260 nm/280 nm absorbance. Integrity of RNA was checked using an RNA 6000 Pico Kit (Agilent) and all samples had RIN>9.6. Whole transcriptome expression profile was determined using a microarray Clariom GoScreen chip (Applied Biosystems, Thermo Fisher Scientific), following the manufacturer's instructions. Briefly, 6 ng of total RNA was used to generate cDNA, then fragmented and labeled cDNA was hybridized to a

Human Clariom GoScreen 384-array plate for 17 h at 45 °C. Arrays were washed, stained and then scanned using the GeneTitan MC Instrument and CEL Intensity files were generated by Affymetrix GeneChip Command Console Software (AGCC, Thermo Fisher Scientific).

2.5. Statistics and Ingenuity Pathway Analysis (IPA) of transcriptomics data

Cell biology and biochemistry data were as mean ± S.D. One-way ANOVA was used to assess the variance within groups of cell tests, and paired and unpaired Student's *t* test was used when appropriate to identify significant differences during the comparison of treatments and phases of treatment (Figure 1A), and a probability error <0.05 was considered significant. The tests were carried out using the statistics package GraphPad Prism version 5.0 (San Diego, CA, USA).

Transcriptomics data were analyzed using the Transcriptomic Analysis Console Software (TAC, Thermo Fisher Scientific) that provides quality control analysis, performs data normalization and summarization based on Signal Space Transformation-Robust Multi-Chip Analysis (SST-RMA) algorithm, and provides a list of differentially expressed genes (Limma Bioconductor package). IPA (QIAGEN) Comparison Core analysis was used to interpret biological functions associated with the transcriptional effects to VE treatments; differentially expressed genes in each treatment were identified applying a gene fold change cut-off of 2 (absolute number) (Krämer et al., 2014). Data were presented as Z-score that is a statistical measure of how closely the actual expression pattern of genes in a dataset compares to the pattern that is expected based on the literature for a particular annotation. Statistical significance (p-value ≤ 0.05) of Z-score data in the different treatments was evaluated by Anova test with eBayes correction of variance, using the "Limma Bioconductor package Version 3.15" (Ritchie et al., 2015).

The effects of VE pretreatments and their correlations with biofunctions and cellular processes (BFs) was studied in comparison with the control test (pretreatment with the vehicle of VE forms, e.g. DMSO, followed by OA treatment), using an activation Z-score (gene fold change >1.7 or < -1.7) and statistical significance was confirmed through Fisher's Exact Test (right-tailed). BFs of interest were selected by "IPA Bioprofiler tool" filtering by anatomical site (liver) and histopathological tissue (hepatic cell lines), and pathway analysis was performed using the "IPA Path Analysis" tool. Only statistically significant (p-value ≤ 0.001) BFs are shown. "IPA Molecule Activity Predictor" tool (MAP) was used to determine up or down modulations of selected biofunctions.

3. Results

3.1. Cell effects of VE formulations and lipotoxicity

Figure 1A shows the VE pretreatment and OA treatment protocol implemented in this study in HepaRG cells.

OA treatment markedly induced the levels of ORO staining in HepaRG cells (Figure 1B, left chart), an indicator of triglyceride biosynthesis and accumulation by lipid droplet formation (Figure 1B, right chart); the pretreatments with all the VE formulations significantly reduced the cellular levels of OA-induced steatosis, and for this effect, a trend toward a higher efficacy of WGO-VE and nVE was observed in comparison with sVE formulation (Figure 1B).

The effect of OA-induced steatosis on lipotoxicity indicators included a significant induction of the levels of apoptosis and necrosis (Figures 1C and 1D, and Suppl. Figure S2), as well as of cellular ROS (Figure 1E and F). Cell viability assessed by MTT test was not significantly modified after OA treatment (not shown), which can be explained by the poor sensitivity of the assay procedure.

The VE formulations significantly reduced the levels of lipotoxicity indicators in OA-treated cells. In the case of apoptosis, their efficacy increased in the order nVE > WGO-VE > sVE, and showed a linear correlation with the reduction of cellular lipid levels (Figure 1D, left chart); furthermore, all the VE formulations almost completely inhibited necrosis (Figure 1D, right chart) and significantly reduced the cellular levels of ROS and their efflux (Figure 1E and F, respectively). In this respect, WGO-VE pretreatment, but not that with the other formulations, stimulated H_2O_2 efflux independently from its combination with OA treatment (Figure 1F).

3.1.1. Transcriptomics data

The OA-induced lipotoxicity of HepaRG cells resulted in the significant modulation of 615 genes, and its combination with VE pretreatments modulated 1224 genes in the case of sVE, 1298 of WGO-VE and 1518 of nVE, respectively; 48 of the significantly modulated genes were in common between nVE and WGO-VE, whereas much lower was the number of responsive genes in common between sVE and nVE ($n = 15$) and sVE and WGO-VE ($n = 7$) (Figure 1G and Suppl. Table S1).

Of these genes, those identified by IPA to be associated with biological functions relevant to liver cell damage and liver disease are shown in Figure 2, Figure 3 and Suppl. Table S2. OA treatment inhibited genes associated with the cellular homeostasis function (Figure 2A), and this effect was significantly prevented by the pretreatment with VE formulations (Figure 2(B, C, D)). However, the three forms of VE showed different effects on the cellular transcriptome. WGO-VE pretreatment inhibited groups of genes associated with liver damage, hepatic steatosis and cancer pathways (including the following gene networks: incidence of tumor, abdominal cancer, digestive organ tumor, formation and frequency of solid tumor). Conversely, sVE inhibited genes associated with hepatic steatosis and liver damage (Figure 2B), while nVE inhibited genes associated with the inflammatory response (Figure 2C).

When IPA Comparison Core analysis was applied to genesets filtered by anatomical site (i.e. liver) and histopathological tissue (i.e. hepatic cell lines) (Figure 3), "Metabolism", "Cancer and tumor progression" and "Cell viability and proliferation" were identified as biofunctions (BFs) and cellular processes (CP) significantly affected by WGO-VE and nVE pretreatments (activation Z-score > 1.7 or < -1.7), whereas any significant associations with BF was retrieved in the case of sVE (Figure 3A and 3B). In this regard, BFs associated with WGO-VE effect included 2 semantic fields (specific biological processes or functions) of metabolism, 9 of cancer and 1 of cell viability, whereas corresponding fields of nVE were 7 of metabolism, 5 of cancer and 5 of cell viability (Figure 3A and 3B). Significant gene modulation effects induced by WGO-VE identified by IPA-MAP were confirmed to include anti-cancer effects, improved cellular homeostasis and inhibition of steatosis (Figure 3C).

4. Discussion

Vitamin E (as α-TOH) is an essential nutrient and fat-soluble antioxidant, involved in the protection of tissues from the harmful effects of oxidative stress and increased lipid peroxidation (recently reviewed in (Brigelius-Flohé, 2021; Galli et al., 2017; Traber and Head, 2021). This function has long been considered particularly important in protecting the liver during the exposure to the free radical-generating agents as CCl_4 (Parola et al., 1992), defects of antioxidant genes such as *GPX4* (Carlson et al., 2016), and lipotoxicity that is the pathogenic cue of non-alcoholic fatty liver disease (NAFLD) (recently reviewed in (Perumpail et al., 2018; Sato et al., 2015; Svegliati-Baroni et al., 2019)). NAFLD is a condition of liver oxidative stress and insulin resistance, that eventually can evolve to non-alcoholic steatohepatitis (NASH) with increased risk of cirrhosis and hepatocarcinoma (Bartolini et al., 2018). According to such hepatoprotective role of VE and to the positive results of large randomized clinical trials, gastroenterology guidelines are now including VE among the first line treatments of NAFLD and NASH (Arab et al., 2014; Chalasani et al., 2012; European Association for the Study of the, European Association for the Study of, & European Association for the Study of, 2016; Watanabe et al., 2015).

However, despite such encouraging pre-clinical and clinical results, mechanistic aspects standing behind the hepatoprotective effects of VE

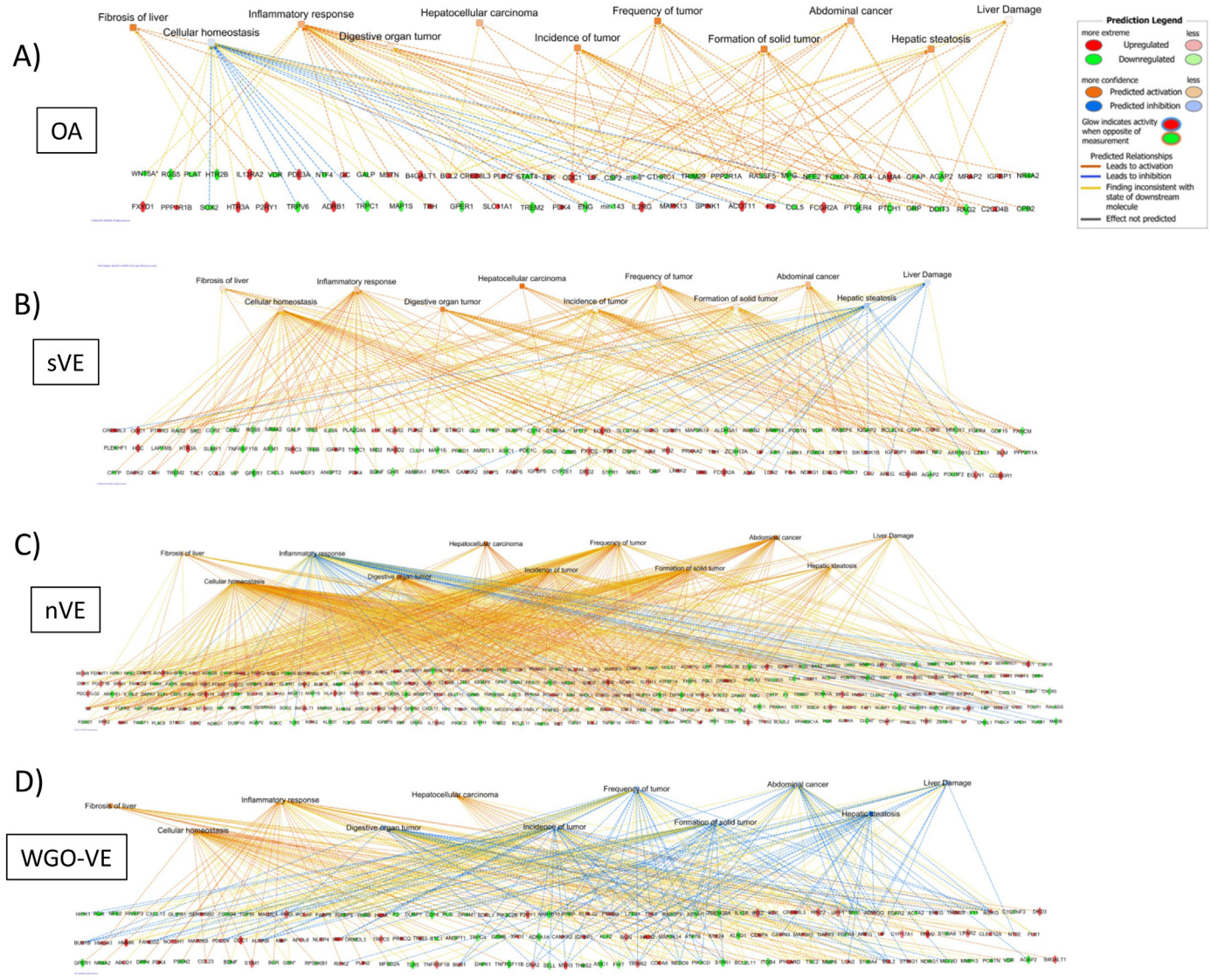


Figure 2. IPA network representation of OA treatment (A) and its combination with sVE (B), nVE (C) and WGO-VE (D) pretreatments. The transcriptional response of each treatment was corrected for the response observed in control cells treated with the vehicle.

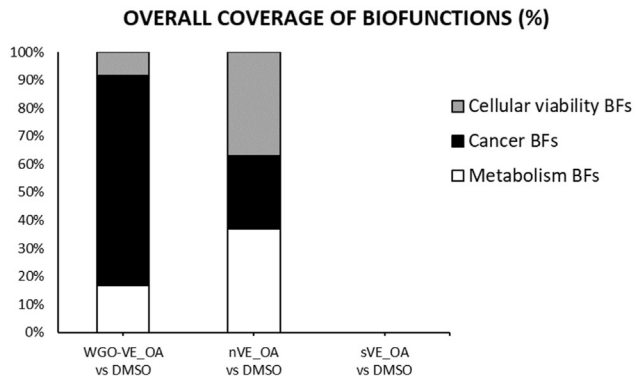
are little understood and specific effects of natural formulations rich in this vitamin, such as WGO-VE, remain unexplored. Besides the role of fat-soluble antioxidant, this vitamin has been demonstrated to influence the expression of genes associated with lipid metabolism and cytoprotection of various cell models and tissues, including hepatocytes and the liver tissue (Podszun et al., 2020; Rimbach et al., 2010; Valastyan et al., 2008).

In this study, we explored the cytoprotective and transcriptional effects of WGO-VE compared with pure forms of natural and synthetic VE, in HepaRG human hepatocytes exposed to OA-induced lipotoxicity. OA is free fatty acid (FFA) that efficiently induces triglyceride biosynthesis and storage of the lipid excess in the lysosomal compartment through lipid droplet formation; important enough is that OA was selected in this study instead of other and more lipotoxic FFA, such as the saturated species palmitic acid and stearic acid (Schmölz et al., 2018), in order to obtain conditions of mild to moderate lipotoxicity (Bartolini et al., 2017; Ricchi et al., 2009) that are compatible with the phenotype of human NAFL (Torquato et al., 2019). This is the earliest step in the development of NAFLD (Svegliati-Baroni et al., 2019) and a relatively benign phase of hepatic lipotoxicity that may benefit of the chemoprotective effects of VE-based nutraceuticals as WGO-VE. In fact, WGO-VE effects were investigated on this *in vitro* model of hepatic lipotoxicity, in chemoprevention mode and important enough was the use of a dose of this VE-rich oil corresponding to 25 nM α-tocopherol. This avoided unwanted steatosis and LD formation during the VE pretreatment phase of the *in vitro* study (Figure 1A). Moreover, this sub-micromolar concentration of the vitamin in the culture medium respects the physiological molar ratio between the vitamin and other lipid components found in plasma or serum. In fact, it is worth noting that, usually, cell culture studies on this vitamin are carried out in the presence of human or bovine sera diluted at least 10 times in culture media, whereas VE is utilized at concentrations that recall those of human plasma (fasting levels range between 15 and 30 μM, while supplemented individuals show levels between 40 and 70 μM) (Bartolini et al., 2021; Hensley et al., 2004); thus the conditions selected for *in vitro* studies do not respect the physiological proportion of the vitamin with plasma lipid concentrations (approx. 10 mM) nor the capability of the cultured cells to host non-toxic loads of vitamin, especially during treatment with FFA that are highly pro-oxidant with respect to VE (P. Torquato et al., 2019). As a consequence, it can be calculated that physiologically relevant concentration of α-tocopherol in the cell culture medium of cells seed at density of 10^4–10^6 cells/mL should be below 100 nM ((Niki, 2021) and references therein), which is compatible with the reported efficacy of submicromolar concentrations of VE and its metabolites in suppressing cellular damage and death program activation induced by different types of stressors (recently reviewed in (Niki, 2021; Schubert et al., 2018) and references therein).

A

	Metabolism BFs	Cancer BFs	Cellular viability BFs
WGO-VE_OA vs DMSO	2	9	1
nVE_OA vs DMSO	7	5	7
sVE_OA vs DMSO	0	0	0

B



C

Field coverage of BFs obtained by previous core analysis	Biofunctions	OA vs DMSO		sVE_OA vs DMSO			nVE_OA vs DMSO			WGO-VE_OA vs DMSO		
		Z-score	p-value	Z-score	p-value	Trend with respect to OA vs DMSO	Z-score	p-value	Trend with respect to OA vs DMSO	Z-score	p-value	Trend with respect to OA vs DMSO
Cancer	Abdominal cancer		9,00E-27		2,00E-44	↓		2,24E-68	↑		5,00E-58	↓
Cancer	Digestive organ tumor		5,00E-25		3,00E-40	↑		1,40E-62	↑		2,00E-52	↓
Cancer	Formation of solid tumor		2,00E-29		4,00E-43	↓		8,21E-67	↓		1,00E-58	↓
Cancer	Frequency of tumor		4,00E-21		2,00E-37	↓		5,53E-63	↓		4,00E-51	↓
Cancer	Hepatocellular carcinoma		4,00E-09		4,00E-07	↑		1,58E-33	↑		1,00E-21	↓
Cancer	Incidence of tumor		4,00E-25		2,00E-38	↓		1,29E-65	↓		4,00E-53	↓
Metabolism/Cell viability	Cellular homeostasis		1,00E-36		2,00E-73	↑		3,46E-85	↑		6,00E-63	↑
Metabolism/Cell viability	Fibrosis of liver		9,00E-08		3,00E-06	↓		1,87E-17	↓		6,00E-09	↓
Metabolism/Cell viability	Hepatic steatosis		7,00E-11		2,00E-22	↓		3,38E-29	↓		5,00E-25	↓
Metabolism/Cell viability	Liver Damage		1,00E-03		4,00E-08	↓		2,80E-10	↓		3,00E-09	↓
Metabolism/Cell viability	Inflammatory response		2,00E-17		4,00E-19	↓		2,08E-48	↓		2,00E-17	↓

Figure 3. IPA Comparison Core analysis to identify semantic fields of interest for liver cell damage and liver disease. A fold change cut-off of 2 was considered for the selected gene. Number of biofunctions modulated by different treatments (A) and graphic representation of percentage of coverage of semantic fields (B). Manual growth of the Biofunctions with respective treatment-induced modulations (C). Only biofunctions statistically significant (p value $\leq$ 0.001) are shown.

Under the experimental conditions of our study, the pretreatment with all the three forms of VE protected HepaRG cells from OA lipotoxicity, as demonstrated by the levels of cellular triglycerides stored in LD, ROS production and cell death by apoptosis and necrosis, that were reduced compared to untreated cells. In this respect, nVE and WGO-VE showed a trend toward a higher protective effect compared with sVE, which can be explained by the different impact on the cellular transcriptome of these formulations. In fact, microarray data indicated that the effect on lipotoxicity parameters of nVE and WGO-VE, but not of sVE, was associated with gene response patterns relevant to liver cell protection and liver disease prevention BF. More in detail, WGO-VE significantly modulated genes associated with liver steatosis and carcinogenesis, whereas nVE modulated genes relevant to liver cell metabolism and viability, whereas sVE modified the expression of genes that were not associated with any of the hepatic BFs selected during IPA (Figure 3 and Suppl. Table S2).

The observed differences between the gene microarray data of pure forms of VE and WGO-VE can be explained by the presence of other bioactive lipids in this VE-rich oil extract; these include phytosterols, polyols, carotenoids and fatty acids that may exert alternative or possibly complementary function with respect to VE in preventing the effects of lipotoxicity (reviewed elsewhere in (Ghafoor et al., 2017; Hassanein and Abedel-Razek, 2009; Kumar and Krishna, 2015)). WGO-VE also contains non-α-tocopherol forms (approx. 30–40% of total tocochromanols present in this oil), and especially β−tocopherol and γ−tocopherol that may play an important role in hepatoprotection and cancer prevention (Jiang et al., 2022). Also, experimental data indicate a higher capability of WGO-VE compared to other forms of VE to stimulate H_2O_2 production and efflux in HepaRG cells (Figure 1F). These findings may lead to hypothesize a higher efficacy for this VE-rich oil compared to pure forms of VE in inducing redox-dependent mechanisms of stress adaption and detoxification (Bartolini et al., 2020; Forman et al., 2014) that are worth investigating further as hepatoprotection mechanisms.

As far as the comparison of sVE with other forms of VE is concerned, our data suggest that the lower efficacy in reducing liver cell steatosis and lipotoxicity parameters, can be explained by the reduced capability of this pure form of VE to affect the expression of genes relevant in these cellular processes. Differences in the gene modulation response to sVE in

comparison to nVE, are expected. In fact, the racemic mixture of sVE is poor in the isomers that bind with high affinity the hepatic α-tocopherol binding protein of liver cells, i.e. primarily the 2R isomer of α-tocopherol and then the 3R isomer that corresponds to nVE (Lauridsen et al., 2002; Traber and Head, 2021). These prevail on the other forms of the raceme in terms of cellular availability and transcriptional activity, and for the possibility to access the liver metabolism, including biotransformation pathways or incorporation into lipoprotein particles for tissue distribution (Galli et al., 2007; Schubert et al., 2018), that are important intersection nodes for the effects of this vitamin and its metabolites to affect lipid metabolism, drug and xenobiotics detoxification, and inflammatory pathways (Bartolini et al., 2017; Marinelli et al., 2020; Parker et al., 2004; Torquato et al., 2017; Willems et al., 2021).

In conclusion, WGO-VE shows hepatoprotective effects in the HepaRG model of OA-induced lipotoxicity comparable to nVE. However, the protection effect of these forms of VE was associated with different and distinctive responses of the cellular transcriptome, highlighting the importance of precision nutrition tools to improve the efficacy of VE in liver protection.

Declarations

Author contribution statement

Desirée Bartolini: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Rita Marinelli: Performed the experiments; Wrote the paper.

Anna Maria Stabile; Tiziana Frammartino; Angela Guerrini; Anna Migni; Linda Zatini; Giada Marcantonini: Performed the experiments.

Stefano Garetto: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Jacopo Lucci: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Mario Rende: Contributed reagents, materials, analysis tools or data.

Francesco Galli, PhD: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data associated with this study has been deposited at “Gene Expression Omnibus” under the accession number GSE195619. [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE195619].

Declaration of interest's statement

The authors declare the following conflict of interests: The Authors Tiziana Frammartino, Angela Guerrini, Stefano Garetto, and Jacopo Lucci are employees of Natural Bio-Medicine SpA, AR, Italy and Innovation & Medical Science Division, Aboca SpA, AR, Italy.

Additional information

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