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Edited by José Antonio Morales-González

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Notice

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Meet the editor

José Antonio Morales-González graduated as a Surgeon Physician from FES-Iztacala, National Autonomous University of Mexico (UNAM). He engaged in Doctoral studies in Biological Sciences at the UNAM. Dr Morales-González has been awarded diverse recognitions: the *Gustavo Baz Prada* Medal by the UNAM, the *Alfonso Caso* Medal for academic merit by the UNAM, and was distinguished by the National System of Researchers (SNI) as National Researcher Level 2 (2017-2020). In addition to this, he has served as director for 16 undergraduate and 46 postgraduate theses. He is the author of 47 internationally published articles, with more than 800 citations to these publications. He is also the editor and coordinator of 28 specialized books and is the author of 41 chapters in specialized books. Dr Morales-González is a Full-time Tenured Professor-Researcher at the Escuela Superior de Medicina, IPN.

Contents

Preface

Oxidative Stress (OS) constitutes an alteration produced by a disequilibrium between the generation of free radicals and the oxidative system, which can contribute to a state of damage, in particular in terms of biomolecules. Similarly, there are natural and synthesized molecules that are capable of inactivating free radicals. These free-radical trappers are classified in groups or families of compounds that are in general called "antioxidants". The main objective of the mechanisms of defense is that of transforming the free radicals into less harmful products or to neutralize their malignancy totally.

Diet and nutrition are very important in the promotion and maintenance of health throughout the entire human lifetime. There has been an attempt to seek, in foods, all of the properties that are of benefit at the time of increasing or maintaining our state of health. There are elements in the diet that, in addition to their nutritional characteristics, are antioxidant agents. Among the most studied of these we find vitamin C, vitamin E, vitamin D, vitamin A, some amino acids, the flavonoids and certain oligoelements. All of these antioxidant elements represent an alternative for the treatment and prevention of Chronic Degenerative Diseases (CDD), which represents a very high morbimortality rate worldwide.

Vitamin E was discovered in the 1920s by Evans and Bishop. Later, it was discovered that vitamin E possesses a lipidic antioxidant effect *in vivo*, in that it blocks the oxidation chain reaction of the lipids that form part of the phospholipids of the cellular membranes. In the present book, *Vitamin E in Health and Disease*, the chapter by Dr Lisa Schmölz et al., The Hepatic Fate of Vitamin E, includes the hepatic metabolism of vitamin E, its storage, release, distribution, and its effects on the metabolism in great detail, as well as its effect on the prevention of diseases, in addition to its role in anti-aging. The chapter by Dr Rusu Anca Elena reports on the effect of vitamin E in patients with hemodialysis, finding a favorable effect of vitamin E in these patients. In a similar manner, the chapter of Drs Rayan Ahmed and Paul W. Sylvester describes g-Tocotrienol, a natural isoform within the vitamin E family of compounds, which displays potent antiproliferative, apoptotic and reversal of epithelial-to-mesenchymal-transition activity against breast cancer, employing treatment doses that have little or no effect on normal cell viability. The chapter by Milka Mileva and Angel S. Galabov describes how vitamin E could be recommended as a reliable agent, indeed as a component in multiorgan flu therapy. Last, Dr Juan José Godina-Nava et al. describe the cytoprotector effect of the 120-Hz electromagnetic fields in early hepatocarcinogene sis.

My congratulations to each of the authors for their chapters in *Vitamin E in Health and Disease* and for their commitment in developing this book.

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Chapter 1

The Hepatic Fate of Vitamin E

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Additional information is available at the end of the chapter

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Abstract

Vitamin E is a lipophilic vitamin and thus is naturally occurring mainly in high-fat plant products such as oils, nuts, germs, seeds, and in lower amounts in vegetables and some fruits. The term "vitamin E" comprises different structures that are classified as tocopherols, tocotrienols, and "vitamin E-related structures." Vitamin E follows the same route in the body like other lipophilic substances. In brief, vitamin E is absorbed in the intestine, packaged into chylomicrons together with other lipophilic molecules, and distributed via lymph and blood in the body. As the liver is the central organ in lipoprotein metabolism, it is also essential for the uptake, distribution, metabolism, and storage of vitamin E. Based on the current knowledge on that field, the physiological, nonphysiological, and pathophysiological factors influencing the hepatic handling of vitamin E, verifying the crucial role of the liver in vitamin E homeostasis, are described.

Keywords: vitamin E, liver, hepatic handling, vitamin E homeostasis, AVED

1. Introduction

Vitamin E is a lipophilic vitamin and thus naturally mainly occurring in high-fat plant products such as oils, nuts, germs, seeds, and in lower amounts in vegetables and some fruits. The term "vitamin E" comprises different structures that are classified as tocopherols (TOH), tocotrienols (T3), and "vitamin E-related structures". However, α-TOH is considered as the most important representative of vitamin E in humans as the central vitamin E metabolizing organ, the liver, discriminates for this form [1]. Notwithstanding the classification as vitamin, the way vitamin E exactly contributes to human health is controversially discussed. Vitamin

E deficiency has been linked to several disease states like ataxia with vitamin E deficiency (AVED) [2, 3] and Alzheimer's disease [4, 5], indicating a role in the preservation of human health. AVED has severe neurological consequences and is caused by a defect in the α-TOH transfer protein (α-TTP); the protein responsible for the discrimination of α-TOH from the other vitamin E forms in the liver [2, 3]. This emphasizes the role of the liver as a central organ in human vitamin E handling. The liver further distributes vitamin E in the body [6] and metabolizes excess vitamin E in order to form products for excretion [6] or presumably to produce activated metabolites of vitamin E as known for other lipophilic vitamins [7]. Given the crucial role of the liver for vitamin E handling, this review aims to summarize the knowledge on the physiological hepatic handling of vitamin E as well as on factors influencing hepatic handling of vitamin E.

2. Physiological hepatic handling of vitamin E

The liver is the central organ of vitamin E handling. While intestinal absorption efficiency is similar for all forms of vitamin E [8], the plasma concentrations of vitamin E forms differ a lot (e.g., 22.1 μM for α-TOH vs. 2.2 μM for γ-TOH [9]). The preference of α-TOH in the human body is mediated by several complex and interacting hepatic mechanisms.

2.1. Hepatocellular uptake of vitamin E

Vitamin E is absorbed in the intestine along with lipids (for details, see [8]) and is packed into lipoproteins. These are transported via lymph or blood toward the liver (via chylomicron remnants, low density lipoproteins (LDL), and high density lipoproteins (HDL) [10, 11]). Different mechanisms facilitate the cellular uptake of vitamin E: (i) via lipid transfer proteins or lipases, (ii) receptor-mediated lipoprotein endocytosis, and (iii) selective lipid uptake [12]. The degradation of chylomicrons to chylomicron remnants by lipoprotein lipase (LPL) seems to be highly important for vitamin E uptake in the liver; when lipolysis of triglyceride-rich chylomicrons by LPL is inhibited, the α-TOH uptake in the liver is diminished [13]. The phospholipid transfer protein (PLTP) mediates the exchange of phospholipids between lipoproteins [14] and is also able to bind α-TOH *in vitro* [15]. PLTP-null mice have lower hepatic levels of vitamin E than the wild-type mice [16]; hence, the transfer of vitamin E between the lipoproteins seems to be important for its effective hepatic uptake. The chylomicron remnants and LDL are taken up by the liver via endocytosis, mainly mediated through the LDL receptor (LDLR) or LDLR-related proteins [6, 17]. In addition, the cholesterol transporter Niemann-Pick C1-like 1 (NPC1L1) is involved in hepatic vitamin E uptake; α-TOH binds to the N-terminal domain of NPC1L1, which mediates α-TOH uptake via endocytosis (mechanism similar to intestinal cholesterol uptake) [18]. The scavenger receptor B type I (SR-BI) is known to mediate the uptake of vitamin E in several tissues (e.g., intestine [19], epithelium [20], and hepatocytes [21]) by channeling the molecules into the cells (shown for cholesterol or triglycerides [22]). Furthermore, the scavenger receptor cluster of differentiation 36 (CD36) is likely involved in hepatic uptake of vitamin E [23].

2.2. Intracellular trafficking of vitamin E

Following its lipophilic nature, vitamin E is transported by intracellular carrier proteins [24]. The intestinally absorbed vitamin E is taken up via endocytosis [25] and follows endosomal fate. Here, the hepatic sorting of vitamin E forms starts as a specific protein, called α-TTP selectively recognizes and preferentially binds α-TOH, which is then extracted from endosomes and transported to the inner leaflet of the plasma membrane [26]. α-TTP is therefore considered to be a "gatekeeper", which discriminates non-α-TOH forms [27] and regulates the plasma concentrations of α-TOH [1]. The affinity of α-TTP to the different forms of vitamin E differs greatly: it is defined as 100% for α-TOH, whereas β-TOH has 38%, γ-TOH 9%, δ-TOH 2%, and α-tocotrienol (T3) 12% affinity to α-TTP [28]. The regular function of α-TTP is crucial, since missense mutations lead to the disruption of α-TOH distribution and the development of a severe degenerative disease, termed AVED [29]. The transfer of α-TOH from endosomes to the plasma membrane is a multi-step process. First, it is speculated whether the ATP-binding cassette transporter A1 (ABCA1) enriches the outer layer of endosomes with α-TOH [30]. The cholesterol transporter NPC1 may also be involved, as a genetic missense mutation of the *NPC1* gene leads to an accumulation of α-TOH in late endosomes [31]. Second, α-TTP extracts the α-TOH from endosomes, and third, α-TTP mediates its transport to the plasma membrane [24]. This process seems to depend on phosphatidylinositol phosphates (PIPs; preferentially $PI(4,5)P_2$ and $PI(3,4)P_2$) in the plasma membrane, as α-TTP binds to them, in turn targeting α-TOH to the plasma membrane and stimulating its release [32]. Chung et al. analyzed the localization of α-TTP depending on the cellular α-TOH concentration [33]. They found (i) perinuclear localization for α-TOH-depleted cells, (ii) a directional transport of α-TOH/α-TTP toward the plasma membrane, when depleted cells were pulsed with a low dose of α-TOH, and (iii) a homogenous cytosolic pattern under long-term and high-dose treatment of cells with α-TOH, which was suggested to be the picture of several α-TOH transport cycles [33]. Furthermore, the authors also postulated a bi-phasic concentration-dependent circulation of α-TTP: the $PI(4,5)P_2$ gradient (low in endosomes and high in plasma membrane) forces the α-TTP-mediated transport of α-TOH toward the plasma membrane, whereas the α-TOH gradient (low in plasma membrane and high in endosomes) triggers the recycling of α-TTP toward the endosomes [33]. It has been proposed that once α-TOH is incorporated into the plasma membrane, it is mediated toward the outer leaflet of the membrane by a flippase, maybe ABCA1, and is then available for the uptake via very low density lipoproteins (VLDL) [34]. For more details on the process, please see Section 2.5 "Release of vitamin E".

2.3. Intracellular storage of vitamin E

Intracellular storage of vitamin E is limited to the lipophilic sites of the cell, which are membranes and lipid droplets [33]. Not much is known about a specific localization of vitamin E accumulation in liver cells, apart from the observation that lysosomal membranes of rat livers seemed to have the highest concentration of all membranes [35–37]. However, it is known that one-third of the total body vitamin E is stored in the liver [38]. Within membranes, vitamin E is thought to stabilize the membrane bilayers due to colocalization with phosphatidylcholine [39] and cholesterol (leading to an association to lipid rafts) [40]. It was further hypothesized

that vitamin E also colocalizes with poly-unsaturated fatty acids (PUFAs) in nonraft domains in order to provide protection from lipid peroxidation [41]. Newly added α-TOH in cell culture enriches in the same organelles as the endogenous α-TOH pool [42]. Hereby, the subcellular content of α-TOH was directly proportional to the lipid content [43].

Our knowledge about the storage of vitamin E in lipid droplets is also limited. It was recently reported that newly endocytosed vitamin E was also found in lipid droplets, thus indicating endosome-lipid-droplet interactions [33].

2.4. Hepatic metabolism of vitamin E

The hepatic metabolism of vitamin E has not been fully characterized. However, the principle steps of vitamin E degradation, that is, the shortening of the side chain without the alteration of the chroman ring, are generally accepted. Hence, the metabolites are classified as α-, β-, γ-, and δ-metabolites according to their respective precursors.

In principle, TOHs and T3s are degraded like long branched chain fatty acids (TOH) or long unsaturated branched chain fatty acids (T3) via β-oxidation in peroxisomes. However, as TOHs and T3s do not bear a terminal carboxy function in their side chain, they are not susceptible to β-oxidation. Hence, the initial and rate-limiting step in vitamin E degradation is the introduction of a carboxy function to the ω-terminus of the side chain. This first step is carried out in the endoplasmic reticulum (ER) of liver cells [44]. Here, two representatives of the cytochrome P450 (CYP) protein family, namely, CYP4F2 [45] and CYP3A4 [46, 47], have been identified to catalyze the initial ω-hydroxylation step. The resulting 13′-hydroxychromanol (13′-OH) is then further metabolized via ω-oxidation, a step that most likely involves alcohol dehydrogenase and aldehyde dehydrogenase [44], leading to 13′-carboxychromanol (13′-COOH). The carboxylated side chain resembles a long branched chain fatty acid that is further degradable via β-oxidation. However, a transport mechanism for the carboxychromanol from the ER to the peroxisomes has not been identified so far. Nevertheless, two cycles of peroxisomal β-oxidation after the activation of α-13′-COOH to the respective CoA ester have been suggested [44], as the peroxisomal β-oxidation system has a higher affinity toward long branched chain fatty acids than the mitochondrial counterpart [48]. The proposed 11′- and 9′-COOH metabolites have indeed been identified in human and mouse samples [49] as well as in a hepatic cell line [45, 50]. Subsequently, three more cycles of β-oxidation are needed to form the final product of vitamin E degradation, namely, carboxyethyl hydroxychromanol (CEHC) or 3′-COOH. These steps, however, are assigned to mitochondrial β-oxidation, as CEHC has solely been found in the mitochondria of hepatic cells [44]. Again, the transport mechanisms of the long-chain metabolites (LCM) (13′- to 9′-COOHs) from peroxisomes to the mitochondria are not known. The respective products for each cycle of β-oxidation (7′-COOH, 5′-COOH, and 3′-COOH) have been identified in different human and murine tissues [49, 51–54] as well as the hepatic cell line HepG2 [45, 47, 51]. Taken together, the hepatic metabolism of vitamin E is characterized by a series of β-oxidation steps after an initial introduction of a carboxy moiety at the ω-terminus of the phytyl-like side chain. The metabolism likely takes place in different cell compartments depending on the enzymatic systems needed for the different degradation steps. However, a concept of vitamin E degradation exclusively in mitochondria cannot be excluded [44]. T3 degradation is believed to follow the same route as TOH degradation but requiring further steps due to the unsaturated side

chain. In line with this assumption is the identification of the respective unsaturated metabolites from 13′-carboxytrienol down to carboxymethylbutadienylhydroxychromanol (CMBenHC) in human and mouse samples [49]. According to these findings, the side chain of the T3 metabolites needs a saturation step before the shortening of the chain. Enzymes involved in the degradation of unsaturated fatty acids like 2,4-dienoyl-CoA reductase and 3,2-enoyl-CoA isomerase were suggested to contribute to the degradation of T3s [55].

2.5. Release of vitamin E

Following the nature of the lipoprotein metabolism, hepatic release of vitamin E is mostly realized via VLDL. Thus, this section will focus on the packaging of vitamin E into VLDL particles, notwithstanding that the mechanism is not well understood. However, hepatic transfer of vitamin E to HDL has also been suggested [56]. Since it was shown that the expression of α-TTP is crucial for the maintenance of plasma α-TOH levels [57, 58] and that the liver is controlling plasma α-TOH levels [59], hepatic α-TTP is likely involved in the incorporation of vitamin E into lipoproteins. This concept is supported by the observation that nascent VLDL particles are preferentially enriched with *RRR*-α-TOH after oral administration of vitamin E ([60, 61]. In contrast, in the liver, no preferential retention of *RRR*-α-TOH was found, indicating that α-TTP is not involved in the delivery of vitamin E to the liver, but in the release from the liver [62]. Hence, efforts have been made to identify the intracellular location of VLDL enrichment with α-TOH mediated by α-TTP [30]. According to the assembly of VLDL, either the rough ER or the Golgi apparatus were assumed. However, the action of α-TTP in these compartments was not confirmed as the nascent VLDL particles contained equal amounts of SRR and RRR α-TOH forms [30]. Further, the inhibition of ER/Golgi action in cells overexpressing α-TTP did not prevent α-TOH secretion [63]. In conclusion, α-TTP is necessary for the hepatic release of vitamin E, but the enrichment of VLDL with *RRR*-α-TOH occurs after exocytosis.

Based on this, the hypothesis of α-TOH uptake by VLDL directly from the plasma membrane was developed. This idea was inspired by the proposed mechanism of the incorporation of free cholesterol into nascent VLDL [64], that is, the spontaneous transfer from membranes to lipoproteins [65]. The hypothesis involves also the α-TTP-mediated trafficking of vitamin E from late endosomes (where vitamin E occurs after cellular uptake and large parts of α-TTP are located [66]) to the plasma membrane. This process might involve ABCA1, which has been shown to transport α-TOH [67] and could thus present vitamin E to α-TTP at the outer leaflet of the endosomal membrane. After the transport to the plasma membrane, a yet unidentified flippase is required to transfer α-TOH to the appropriate site of the membrane for uptake by nascent VLDL [30]. This hypothesis is supported by findings of Chung et al. [33], which provided a model of α-TTP-facilitated trafficking of vitamin E from endosomes to the plasma membrane (the reader is referred to Section 2.2 "Intracellular trafficking of vitamin E"). Taken together, the release of α-TOH from hepatocytes depends on vesicular transport [21, 31, 63, 68, 69], but is independent from ER or Golgi [63]. Hence, lipoproteins are not loaded with TOH during their intracellular assembly, but rather after exocytosis, a mechanism is required for the presentation of α-TOH at the plasma membrane. Evidence has been provided that the trafficking of α-TOH to the plasma membrane is realized via α-TTP which is located at recycling endosomes in hepatocytes [33]. However, the mechanism of

the loading of lipoproteins with α-TOH from the plasma membrane has not been elucidated yet, although the involvement of ABC transporters has been suggested [56, 67, 70]. However, ABC transporters are fueling HDL particles, which is in contrast to the assumption that the hepatic release of α-TOH is mediated via VLDL. In turn, two explanations have evolved: first, α-TOH translocates spontaneously from the membrane to VLDL like free cholesterol [65], and second, α-TOH is transported to HDL via ABCA1 and is then spontaneously transferred to VLDL [71]. However, both hypotheses need evaluation. A recent report on the self-assembly of α-TTP to form nanoparticles and transport vitamin E to tissues protected by endothelial barriers like the brain [34] opens another possible way for the distribution of vitamin E throughout the body starting from the liver.

3. Factors influencing hepatic handling of vitamin E

3.1. Effects of vitamin E

3.1.1. Intracellular handling of vitamin E

Key factors in the hepatic handling of vitamin E have been outlined in the previous sections. This section will focus on the action of vitamin E on its own intracellular handling. As indicated above, the key enzyme for the intracellular trafficking of vitamin E is α-TTP, and the rate-limiting enzymes in vitamin E metabolism are CYP4F2 and CYP3A4. Hence, we will here focus on the known actions of vitamin E on these key players.

The key protein of the hepatic handling of vitamin E is α-TTP, with its implications in cellular trafficking, metabolism, and release of vitamin E. Hence, several studies have been conducted to elucidate a possible feedback regulation of α-TTP in response to vitamin E intake, resulting in alterations of the metabolism or the distribution of the vitamin. In principle, research is focused on three levels of regulation: mRNA expression, protein expression, and stabilization of α-TTP protein. However, contradictory results from rodent models have been reported. Fechner et al. found that hepatic α-TTP mRNA expression was strongly induced in rats depleted from vitamin E for 5 weeks after the intake of a TOH-supplemented diet for 24 h [72]. However, rats fed a vitamin E-depleted diet, control diet, or vitamin E-enriched diet for 20 weeks showed upregulation of α-TTP mRNA when vitamin E is deprived, but a downregulation when vitamin E was repleted. Hepatic α-TTP protein levels were comparable for depletion and control, but lowest in rats fed the repleted diet [73]. A similar study reported no differences in hepatic α-TTP mRNA levels of rats fed either a control diet or a diet rich in or low in vitamin E. However, in contrast to the aforementioned study, downregulation of α-TTP protein was reported in the vitamin E-depleted group, while high vitamin E intake did not alter the levels compared to control [74]. The lack of an effect of a vitamin E deficient diet for 290 days on hepatic α-TTP mRNA levels was also reported in another rat model [75]. In line with this, subcutaneous injection of vitamin E for up to 18 days did not alter α-TTP protein levels in rats [76]. However, mice fed a diet rich in vitamin E showed 20% higher hepatic α-TTP protein levels than mice fed a low vitamin E diet [77]. Taken together, some studies

report elevated α-TTP levels due to a higher intake of vitamin E [72, 77], but some revealed no effect [74–76] or even lower levels [73]. Hence, further studies are needed to clarify the role of vitamin E in the regulation of α-TTP. In addition, an *in vitro* study suggested that vitamin E does not regulate α-TTP at the level of gene expression, but stabilizes α-TTP at the protein level upon binding and thus protects the protein from degradation, leading to higher α-TTP protein levels [78]. Reports on the hepatic mRNA levels might thus be of minor importance for the interpretation of the contribution of vitamin E to α-TTP action; however, the findings on α-TTP protein expression are also inconsistent.

The rate-limiting enzymes of vitamin E metabolism are CYP4F2 and CYP3A4. The latter was reported to be under transcriptional control of pregnane-X-receptor (PXR) [79, 80]. Hence, vitamin E might regulate its metabolism by binding to PXR and subsequent alteration of the expression of the enzymes involved in the first catabolic step. Indeed, studies using cells transfected with reporter genes provided evidence for an activation of PXR by different vitamin E structures (i.e., TOHs, T3s, and metabolites) [81, 82]. Interestingly, α-, δ-, and γ-TOH as well as α- and γ-T3 activated PXR in HepG2 liver cells transfected with human PXR and chloramphenicol acetyl transferase linked to two PXR responsive elements [81], while α- and γ-TOH as well as their metabolites α- and γ-CEHC did not in transfected colon carcinoma cells [82]. However, the LCM α-13′-COOH activated PXR in the latter cellular system and so did γ-T3 [82]. This finding implicates that the LCM of TOH are the responsible mediators of reported TOH actions via PXR. Hence, the findings in hepatic HepG2 cells [81] might be due to a higher catabolic rate of TOH and in turn the more efficient formation of the LCM than in colon cells. However, these findings were made in artificial cellular reporter systems and might not resemble the actual (hepatic) situation *in vivo*. Further, the specificity of PXR might depend on the species, as γ-T3 (the vitamin E form that activated PXR in both of the aforementioned studies) fails to bind murine PXR [83]. However, results obtained *in vivo* support the regulation of Cyp3a11 (the murine orthologue of CYP3A4) by vitamin E via PXR. Mice supplemented with α-TOH show elevated hepatic expression of Cyp3a11, while their PXR-deficient counterparts as well as mice with humanized PXR showed no upregulation of Cyp3a11 in response to α-TOH [84]. The same finding was made for Cyp4f13, the murine orthologue of CYP4F2, in this model [84]. These findings suggest that both enzymes are under the control of PXR and murine, but not human PXR is susceptible to α-TOH (or its metabolites as outlined above). Further studies reporting upregulation of hepatic Cyp3a in rodent models with α-TOH supplementation support this finding [76, 83, 85]. Interestingly, in these studies, γ-TOH and γ-T3 had no effect on Cyp3a expression [83, 85], supporting the suggested specificity of murine PXR for α-TOH. In line with this, γ-TOH did not alter the expression of Cyp4f13 in mice [85]. However, subcutaneous application of α-TOH in rats did not induce Cyp4F2 levels [76], which is in contrast to the above mentioned induction of Cyp4f13 in mice via PXR [84]. The reported induction of CYP4F2 activity in HepG2 cells by α-TOH further complicates the interpretation of the data on the effect of vitamin E on CYP4F2 [45]. Taken together, there is evidence for the regulation of CYP4F2 and CYP3A4 via PXR by vitamin E in the human liver. However, several aspects need further clarification, for instance, species and vitamin E isoform specificity of PXR, the regulation of CYP4F2 by vitamin E or the relevance of the α-LCM as true mediators of α-TOH effects via PXR.

3.1.2. Vitamin E intake

Several key enzymes determine the rate of vitamin E catabolism (the reader referred to Section 2.4 "Hepatic metabolism of vitamin E") and, as outlined in the previous section, there is evidence that vitamin E in general might regulate its own metabolism. However, there are differences in the ability to regulate the metabolism depending on structural properties of the vitamin E isomers (i.e., methylation of the chroman ring, saturation of the side-chain, and stereochemistry). In principle, high intake of vitamin E, independent from the isomer, leads to enhanced formation of the respective metabolites [49]. However, the catabolic rates of the different forms of vitamin E clearly differ: the γ-isoforms are more susceptible to metabolization than the α-isoforms. Subjects supplemented with γ-T3 and α-T3 (125 mg or 500 mg) showed four to six times higher urinary excretion of the catabolic end product γ-CEHC and an induction of α-CEHC only after high dose (500 mg), but not after low dose supplementation (125 mg) [86]. In line with this, equimolar supplementation with 50 mg of α- and γ-TOH leads to a twofold increase of plasma γ-CEHC, but no alterations in α-CEHC [87]. These data indicate that there might be a threshold for the intake of α-TOH and α-T3 (or plasma levels, respectively) that needs to be exceeded to accelerate catabolism of α-TOH and α-T3 to form α-CEHC, as suggested by Schuelke et al. [88]. Interestingly, already in 1985, Handelman et al. reported that high α-TOH levels in human plasma are related to low γ-TOH levels [89]. After supplementation of α-TOH, the plasma α-TOH levels were, as expected, twofold to fourfold higher, but the γ-TOH level decreased to between one-third and one-half of the initial level [89]. Hence, α-TOH intake seems to boost γ-TOH catabolism. Supporting data were generated in a rat model, where the combined supplementation of α- and γ-TOH leads to higher excretion of γ-CEHC than the supplementation of γ-TOH alone [90], as well as the reported stimulation of γ-TOH catabolism by α-TOH in HepG2 liver cells [91]. Although the underlying mechanisms are not fully unraveled, there is evidence that α-TOH induces the activity of enzymes involved in the metabolism of vitamin E, leading to the degradation of non-α-forms, while α-TOH remains protected (please refer to Section 3.1.1 "Intracellular handling of vitamin E").

3.2. Effects of other compounds

3.2.1. Intake of sesamin

Sesamin is a lignan, a group of natural compounds derived from vegetable sources, like sesame seeds [92]. Sesamin is known as a natural inhibitor of the metabolism of TOH [93–97]. The cell regulatory actions of sesamin have been initially investigated in *in vitro* models, where Parker et al. showed that sesamin acts as a selective inhibitor of CYP3A4, an initial enzyme of TOH metabolism [46]. In this study, the authors compared the inhibitory potential of sesamin on TOH metabolism in human HepG2 cells to the well-characterized CYP3A4 inhibitor ketoconazole. HepG2 cells were treated with one of the mentioned compounds in combination with either 25 μM α-TOH or 25 μM γ-TOH. Afterwards, the concentration of the corresponding CEHC was determined as a marker for TOH metabolism in cell culture media. It became apparent that ketoconazole (1 μM) and sesamin (1 μM) inhibited the formation of α- and γ-CEHC. This result provides evidence that sesamin is able to modulate TOH metabolism via the inhibition of

CYP3A4 [46]. In addition to the *in vitro* data, Uchida and coworkers investigated the inhibitory effects of sesamin on vitamin E metabolism in rats. Vitamin E-deficient rats (vitamin E free diet for 4 weeks) were treated with 50 mg/kg *RRR*-α-TOH alone or in combination with 200 g/kg sesame seeds [95]. Next, the concentration of α-TOH in different tissues as well as the urinary excretion of α-CEHC was measured. The urinary excretion of α-CEHC in the sesamin group was significantly lower compared to the α-TOH control group. Further, the combination of α-TOH and sesamin provoked a significant increase of hepatic α-TOH concentrations compared to α-TOH treated animals [95]. These observations have been confirmed in other animal studies [93, 94]. Beside the investigations in animal models, there are also a few results originating from studies in humans. In 2004, Frank and colleagues used muffins enriched with sesame oil (94 mg sesamin/muffin) or corn oil (control) to investigate the effect of a single dose sesamin application on urinary excretion of γ-CEHC as well as blood levels of γ-TOH in 10 healthy volunteers [97]. Both, control and intervention group, received the muffins together with a capsule containing deuterium-labeled γ-TOH (50 mg) in a crossover design. Blood and urine samples were collected over 72 hours after the application of the muffins and capsules. While the urinary excretion of γ-CEHC was significantly lowered, the sesamin treatment did not affect γ-TOH concentrations in blood compared to the corn oil control group [97]. Unfortunately, the study does not provide data on the elevation of the hepatic γ-TOH concentration in response to the reduced urinary excretion of γ-CEHC. Taken together, *in vitro* and *in vivo* studies provide evidence that the dietary intake of sesamin leads to an increase of the hepatic concentration of TOH via the inhibition of vitamin E metabolism, but further experiments are needed to characterize the interaction of sesamin and vitamin E metabolism in more detail.

3.2.2. Pharmacological activation or inhibition of CYP3A4

The pharmacological modification of the enzymatic activity of CYP3A4 represents an effective way to influence vitamin E homeostasis in the human body. Mechanistically, the direct or indirect interference of vitamin E metabolism is usually just a side effect of the pharmacological inhibition or induction of CYP3A4 by various chemical compounds. Thus, it is not surprising that the first evidence for the involvement of CYP3A4 in vitamin E metabolism was provided in an experimental subset using ketoconazole as a specific inhibitor for CYP3A4 [46, 98]. In HepG2 liver cells, different concentrations of ketoconazole (1 mmol/l or 0.25 mmol/l) inhibited the metabolic conversion of γ- and δ-TOH (25 μmol/l cell culture media) to γ- or δ-CEHC by almost 90% [46]. This finding has been confirmed by the reproduction of the same experiment with sesamin, the natural inhibitor of CYP3A4, revealing comparable results [46]. The inhibitory effect of ketoconazole on vitamin E metabolism has further been observed in an *in vivo* model. Here, rats were supplemented with ketoconazole (50 mg/kg body weight) together with α-TOH (10 mg/kg body weight), γ-TOH (10 mg/kg body weight) or mixture of different T3s (29.5 mg/kg body weight). Ketoconazole significantly reduced the catabolism of all applied vitamin E forms resulting in impaired urinary excretion of the respective CEHCs [99]. Beside its inhibition, the pharmacological induction of CYP3A4 represents another way to modulate vitamin E metabolism. Birringer and coworkers demonstrated that 50 μmol/L rifampicin, an inducer of CYP3A4 activity [100], induced the degradation of all-*rac*-α-TOH

in HepG2 cells fivefold [47]. In this study, the cell culture medium has been preconditioned with 100 μmol/L α-TOH for 10 days, as the standard medium was deficient for α-TOH [47]. Further, an indirect approach for the modulation of vitamin E metabolism via the modification of CYP3A4 expression could be realized by triggering PXR, a nuclear receptor that regulates the expression of metabolic enzymes and transporters involved in the metabolism of xenobiotics and endobiotics [101, 102]. Landes and coworkers showed that γ-T3 as well as rifampicin acts as PXR agonists, thus upregulating CYP3A4 mRNA expression in HepG2 liver cells [81]. Given the fact that enhanced mRNA expression of CYP3A4 results in enhanced enzymatic activity, the stimulation of PXR by various pharmacological agonists or antagonists could also modulate the hepatic metabolism of vitamin E. In summary, the direct or indirect regulation of CYP3A4 by various pharmacological means represents an effective way to modify the hepatic vitamin E metabolism.

3.3. Nonmodifiable factors influencing handling of vitamin E

The handling of vitamin E is also influenced by nonmodifiable factors. These are aging, gender, and individual genetics. Published data in this area are sparse but interesting.

3.3.1. Aging

The aging process is characterized by nine hallmarks: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication [103]. In particular, the mitochondrial dysfunction leads to higher formation of reactive oxygen species (ROS) and enhanced oxidative damage [104]. Both processes can be diminished by the antioxidant function of vitamin E [105]. Consequently, two questions arise: (i) can vitamin E modulate the aging processes or prevent age-related diseases? This has been subject of several reviews [106–109]. (ii) And how is the concentration, distribution, and function of vitamin E modulated by the aging process? In humans, age-dependent changes of α-TOH plasma concentrations are known. In healthy aged humans, the α-TOH plasma concentrations are higher than in younger individuals [110–113]. However, this might be due to the age-related increase of plasma cholesterol concentrations, as the age-related increase in α-TOH plasma concentrations disappear after adjustment for cholesterol plasma concentrations [112] or serum lipids [113]. Traber et al. suggested that α-TOH plasma concentrations are more dependent on control mechanisms for plasma lipids rather than on α-TOH absorption [113]. Hospitalized elderly patients [114] as well as older persons with cognitive impairments (dementia or Alzheimer's disease [115, 116]) have low α-TOH plasma concentrations [117]. However, an unfavorable nutrient status of the hospitalized patients was discussed as the cause of the lower α-TOH plasma concentrations.

Several studies analyzed the age-dependent changes of α-TOH tissue concentrations and handling in mice [37, 117–119] and rats [120]. In brain [37, 117, 118] and kidney [37, 117], epididymal adipose tissue [117] and aortic vessel wall [120], a consistent increase in α-TOH was found with age. In old rats, however, an age-dependent increase in intestinal absorption was found [121]. This was considered as a "*self-protective age-dependent adaption*" [120], which

is thought to counteract increased oxidative stress during aging. In the liver and heart, however, data are conflicting: while some found increased concentrations [37, 119, 120], Takahashi et al. found decreased values [117]. Two studies also analyzed the age-dependent regulation of genes, known to be involved in vitamin E handling, which are α-TTP, ABCA1, and Cyp4f14 (murine orthologue of CYP4F2) [117] as well as NPC1, NPC2, and LPL [37]. Takahashi et al. found increasing (mice with the age of 3–12 month) and then decreasing (12—24 months) α-TTP protein levels in the liver, while mRNA expression was stable over age [117]. Overall, Cyp4f14 mRNA expression decreased during aging (60% decrease in mRNA expression at the age of 24 months compared to the age of 3 weeks), while ABCA1 mRNA expression slightly increased (20% in the same age range as measured for Cyp4f14) [117]. The authors concluded that the age-related changes of hepatic α-TOH levels cannot be explained by the metabolism of α-TOH via Cyp4f14. König et al. analyzed protein expression in kidney tissue or its lysosomal membranes and found a significant decrease of NPC1 and NPC2, but a prominent increase in LPL (361% compared with the tissue from younger mice) [37]. The increased expression of LPL may explain the accumulation of α-TOH in aged mice. Furthermore, NPC1 and NPC2 may be responsible for the transport of α-TOH from the endosomes to the cytosol [69] and their reduced expression may explain the accumulation of α-TOH in lysosomal membranes [37]. In summary, there are age-dependent changes in α-TOH tissue and plasma concentrations and also in the expression of genes responsible for vitamin E handling; however, the underlying regulatory processes are not unraveled completely yet.

3.3.2. Gender

The sex-dependent differences in vitamin E handling were described recently by Schmölz et al. [6] and will be summarized here briefly for humans only. While intake of vitamin E in total is higher in men than in women [122], the intake per kcal is higher for women than for men [123]. The absorption of α-TOH seems not to be influenced by sex, but is mainly regulated by downstream regulatory processes (likely by hepatic sorting or metabolism) [113]. The data on serum concentrations of vitamin E are inconsistent: while some researchers reported elevated α-TOH serum concentrations for women compared to men [124, 125], others found contradictory results [123]. Sex-dependent regulation of vitamin E metabolism is specific for the different forms of vitamin E. Women degrade γ-TOH to a higher degree than men, while the metabolism of α-TOH seems to be independent [87]. Two mechanisms may be relevant for sex-dependent regulation of vitamin E metabolism: the hormonal status of individuals and the activation of the CYP enzymes involved in vitamin E metabolism [6]. Further studies could illuminate gender-specific differences in more detail. In the light of the discovery of vitamin E as a factor that limits female fertility, this is of special interest.

3.3.3. Genetics

The influence of genetics on vitamin E handling was summarized in detail in a recent review (for more details, please see [6]). Therefore, only a short overview will be provided here. Interindividual differences in the handling of vitamin E can be caused by individual genetic constitutions. Polymorphisms in genes, which are responsible for vitamin E handling such as

CYP4F2 [126], NPC1L1 [127], and CD36 [128] are likely to contribute to variations in vitamin E status. The best-studied gene in this context is α-TTP, as its genetic variability may cause AVED. Two genetic variants are known, which are located in or nearby the proposed tocopherol-binding domain and cause reduced α-TOH serum concentrations [129]. Furthermore, mutations in the promoter region of α-TTP (with increased or decreased activity) were also reported [130]. In summary, vitamin E handling is influenced by several mechanisms, one of which is the variability of genes involved in these processes. This might held responsible for interindividual differences in vitamin E serum concentrations.

3.4. Pathophysiological factors influencing handling of vitamin E

3.4.1. Nonalcoholic fatty liver disease and nonalcoholic steatohepatitis

Nonalcoholic fatty liver disease encompasses a histological spectrum ranging from simple steatosis to nonalcoholic steatohepatitis (NASH). NASH is a clinical symptom characterized by a pattern of steatosis, inflammation, and hepatocyte ballooning, which can result in the development of cirrhosis and liver cancer [131]. Although the molecular mechanisms of NASH development remain poorly understood, studies provide evidence for a critical role of oxidative stress together with an impaired antioxidative response [132, 133]. In line with this, Erhardt and coworkers observed significantly lower plasma levels of α-TOH and other antioxidants in NASH patients compared to healthy controls [134]. Given the fact that an induction of CYP3A4 or CYP4F2 results in decreased vitamin E concentrations in the human body, it has been expected that NASH leads to an enhanced activity or expression of these enzymes. Thus, Woolsey and coworkers investigated the enzymatic activity as well as the mRNA expression of CYP3A4 in NASH patients [135]. The authors used liver biopsies for mRNA analyses and determined the concentration of 4β-hydroxycholesterol in plasma as an endogenous biomarker for CYP3A4 activity. Interestingly, NASH patients showed a 37% reduced enzymatic activity of CYP3A4 as well as a 69% lower CYP3A4 mRNA expression compared to healthy controls [135]. Unfortunately, there is no further data on the activity or the expression of CYP4F2 in NASH patients. However, Athinarayanan and coworkers investigated the influence of two different CYP4F2 genotypes (V433 M and W12G) on vitamin E plasma concentrations in NASH patients [136–138]. The V433 M genotype was associated to higher baseline levels of vitamin E, indicating lower enzymatic activity compared to the W12G genotype [136–138]. Thus, the authors hypothesized that the W12G genotype in NASH patients could explain the lower vitamin E plasma concentrations. However, this hypothesis has been disproved by the finding that the vitamin E plasma concentrations of NASH patients did not differ between the two CYP4F2 genotypes [136–138]. Based on the available data, CYP4F2 and CYP3A4 seem to have no influence on vitamin E plasma concentrations during the NASH development. Next to the CYPs, α-TTP could also be involved in a potential mechanism explaining the observation of Erhardt and coworkers mentioned above. In line with this, Ban and coworkers used a rat model to investigate whether an exposure to hyperoxia (>95% O_2 for 48 h), an established stimulus for ROS production [139], could alter the expression of hepatic α-TTP [140]. Indeed, hyperoxia decreased the expression of α-TTP mRNA in rat liver, while α-TTP protein expression remained unchanged [140]. As oxidative stress and ROS

formation are crucial factors for NASH development, lowering α-TTP expression by ROS could explain the lower vitamin E levels in NASH patients. In summary, the concentration of vitamin E and other antioxidants is reduced in NASH patients by yet not fully understood molecular mechanisms, potentially involving α-TTP. Nevertheless, recent human intervention trials provide evidence that vitamin E treatment could improve primary NASH outcomes (i.e., steatosis, inflammation, hepatocellular ballooning, and fibrosis) [137, 138].

3.4.2. Cancer

The current data on vitamin E as a potential agent for cancer therapy are inconsistent. While *in vitro* and early epidemiological studies provided evidence for cell growth-inhibiting, antiproliferative and pro-apoptotic effects of vitamin E in cancer treatment [141–145], more recent investigations reported contradictory results [146–148]. These findings were further sustained by the "Selenium and Vitamin E Cancer Prevention Trial (SELECT)," a randomized intervention study to determine the long-term effect of a supplementation of vitamin E (400 IU/d all-*rac*-α-tocopheryl-acetate) and selenium (200 μg/d L-selenomethionine) on the risk of prostate cancer in healthy men. Interestingly, the authors observed an increased incidence for prostate cancer in subjects supplemented with vitamin E [149]. Beside the investigations on beneficial effects of vitamin E in cancer therapy, almost nothing is known about the influence of cancer on human vitamin E homeostasis. An early study by Knekt, who investigated the association of vitamin E serum concentrations and the risk for different types of female cancer, showed an inverse relation between α-TOH serum concentrations and cancer risk [150]. Thus, women with the lowest α-TOH levels were at enhanced risk for cancer compared to those with higher α-TOH levels. Indeed, this association was restricted to cancer outcomes in tissues and organs, which were not exposed to estrogens [150]. Thus, Knekt hypothesized that low vitamin E levels could represent a potential risk factor for several, but not all types of cancer [150]. Nevertheless, the molecular mechanisms underlying this impairment of vitamin E serum concentrations in cancer patients remain unclear. The enhanced metabolic conversion of vitamin E might represent a mechanistic explanation. In line with this, investigations of tissues from cancer patients showed elevated expression of CYP3A4 [151] and CYP4F2 [152], the two major enzymes of vitamin E catabolism. Unfortunately, vitamin E serum concentrations have not been determined in these studies. Further, *in vitro* studies provided evidence that cancer also affects transporters for vitamin E, such as the tocopherol-associated protein (TAP) [153]. Tissue samples from prostate cancer patients showed significantly lower TAP mRNA expression compared to healthy controls, indicating that cancer may affect the intracellular transport of vitamin E. In addition, the overexpression of TAP in prostate cancer cells leads to a significant reduction of cell growth, while a TAP knockdown by small interfering RNA increased their growth [153]. Interestingly, these effects appeared without additional vitamin E treatment, indicating that TAP not only mediates vitamin E transport but also functions as a vitamin E-independent tumor suppressor gene [153]. In summary, the promising cancer preventive effects of vitamin E shown *in vitro* have not been confirmed in recent *in vivo* trials. Nevertheless, cancer could probably be associated with reduced vitamin E concentrations in the human body, because of an enhanced vitamin E catabolism and/or the alteration of its intracellular transport. However, further investigations are required to validate these results.

3.4.3. Disorders of lipoprotein metabolism

After its intestinal absorption, the transport of vitamin E, including its transfer to and its export from the liver as well as the subsequent distribution of vitamin E in the human body, strictly depends on different lipoproteins [7]. Thus, disorders of the lipoprotein metabolism can lead to disturbances of vitamin E homeostasis. Abetalipoproteinemia or Bassen-Kornzweig syndrome is a rare form of neurodegenerative ataxia with a strong impact on the hepatic handling of vitamin E. Abetalipoproteinemia is caused by mutations in the gene encoding for the microsomal triglyceride transfer protein (MTP), which is required for the assembly and secretion of the apolipoprotein B (apoB) forms in the liver and the intestine [154]. The apoB forms are the primary apolipoproteins associated to chylomicrons or VLDL, IDL, and LDL, respectively, and are thus essential for the distribution of vitamin E in the human body [7, 155]. As a result of the disturbed intestinal absorption and hepatic excretion of all lipid soluble molecules, patients with abetalipoproteinemia show vitamin E deficiency as well as low serum concentrations of cholesterol and triglycerides [156]. Next, the hepatic handling of vitamin E can be affected by familial hypobetalipoproteinemia. This lipoprotein disorder is caused by mutations in the *APOB* gene, leading to disturbances of translation of the apoB proteins and/or impaired secretion of VLDL [157]. Thus, familial hypobetalipoproteinemia displays the same clinical features as abetalipoproteinemia. In summary, lipoprotein disorders exert clear impact on the hepatic and systemic handling of vitamin E.

3.4.4. Other relevant pathophysiological factors

AVED is a neurological disorder, which has for the first time been described in a 12-year-old boy with cerebellar ataxia and low serum vitamin E concentrations. Interestingly, the boy showed no lipid malabsorption or a lack of lipoproteins, like it has been observed in abetalipoproteinemia [158]. Subsequent studies identified a mutation in the *TTPA* gene, the gene encoding for α-TTP, as the disease causing factor [159]. Thus, AVED patients have impaired expression of α-TTP, leading to impaired incorporation of vitamin E (α-TOH) into VLDL as well as a higher metabolic conversion and excretion of vitamin E [154]. In addition, AVED patients show very low plasma vitamin E concentrations together with normal absorption rates for vitamin E in the absence of intestinal malabsorption and abetalipoproteinemia [2, 154]. In summary, AVED represents a clinical condition that includes altered hepatic handling of vitamin E without affecting lipoprotein homeostasis.

4. Conclusion

In the last decades of vitamin E research, the liver appeared as the central organ for the uptake, distribution, metabolism, and storage of vitamin E. Thus, it is also a starting point for various strategies for the modulation of the vitamin E homeostasis. Based on current knowledge, we identified physiological, nonphysiological as well as pathophysiological factors influencing the hepatic handling of vitamin E, verifying the crucial role of the liver in vitamin E homeostasis (a brief schematic overview is provided in **Figure 1**). Nevertheless, further studies

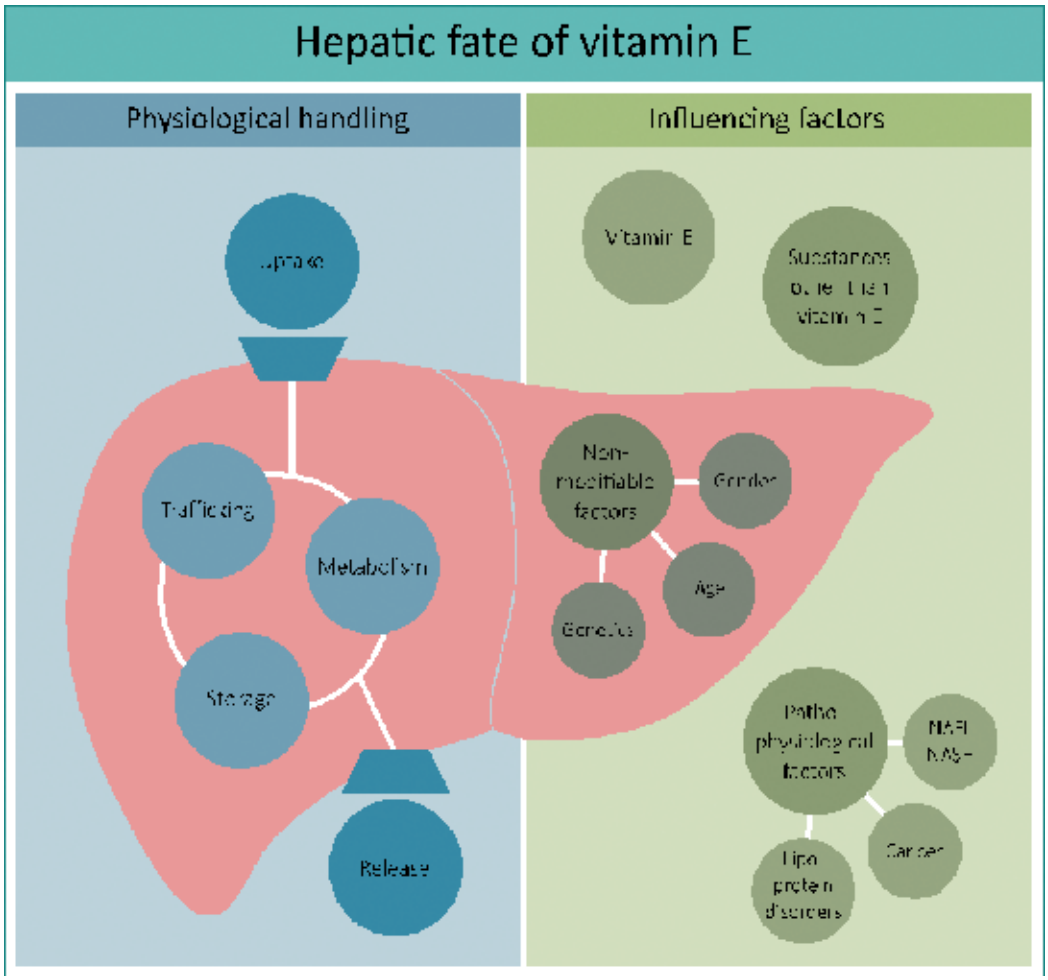

Figure 1. The crucial role of the liver in vitamin E homeostasis.

are needed to unravel the molecular mechanisms underlying the described disturbances of hepatic vitamin E handling by various factors.

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Chapter 2

Vitamin E in Hemodialysis Patients

Anca Elena Rusu

Additional information is available at the end of the chapter

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Abstract

End-stage renal disease patients treated with hemodialysis are characterized by a special diet, increased oxidative stress, cardiovascular morbidity and mortality, as well as many other complications such as inflammation-malnutrition syndrome, muscle cramps, and anemia. Worldwide efforts are focused on reducing hemodialysis complications to increase survival in these patients. In vitro and in vivo studies proved that vitamin E has many beneficial effects such as: decreases reactive oxygen species synthesis, improves antioxidant defense system, inhibits lipids peroxidation and reduces atherosclerosis, and ameliorates anemia treatment. Mechanisms of action are complex and not fully understood. However, there are particularities in regards of vitamin E intake, metabolism, and clearance in patients treated with hemodialysis. Supplementation of vitamin E in these patients has been intensively studied, and it is still under debate. Oral administration and vitamin E-coated membranes for dialysis have been tried. Clinical practice guidelines tried to underline when and how much vitamin E to be given to be safe and cost-beneficial. The current chapter aims to synthesize all these issues.

Keywords: hemodialysis, vitamin E deficiencies, oxidative stress, anemia, cardiovascular diseases, vitamin E supplementation

1. Introduction

Hemodialysis (HD) patients are a special population with many particularities in regard of nutrition, metabolism, inflammation and oxidative stress, morbidity, and mortality. Contacts with extracorporeal dialysis membranes generate inflammation and oxidative stress and the entire cascade of complications. Intake, metabolism, and clearance of many nutrients are limited or disturbed, and vitamin E is one of them. On the other hand, beneficial effects of vitamin E come to recommend its supplementation in order to limit oxidative stress and other complications in hemodialysis patients.

2. Particularities of HD patients

- dietary restrictions
- increased oxidative stress (as displayed in **Figure 1**)
- increased inflammation markers and malnutrition inflammation syndrome
- muscle cramps during dialysis
- disturbances in lipid profile
- increased cardiovascular risk

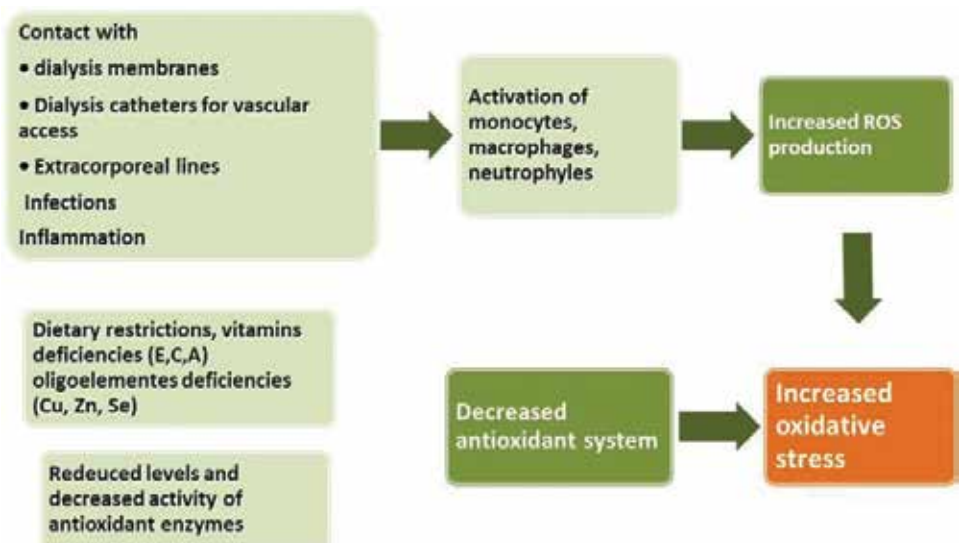

Figure 1. Oxidative stress in hemodialysis patients.

3. Status of vitamin E in HD patients

The level of vitamin E in HD patients is influenced by dietary intake, particularities in metabolism and clearance. The plasma level of vitamin E is usually normal, even though there are studies that found predialysis low levels of alpha-tocopherol. However, the number of patients included was small and similar results have not been identified in other larger studies. Gastrointestinal disturbances in uremic patients might lead to poor absorption and this could be an explanation for low plasma levels of vitamin E identified in some HD patients, while increased consumption is another one [1–4].

Profile of vitamin E status in these patients is as follows:

- limited intake
- not cleared by dialysis

- metabolism disturbances
- normal/reduced plasma levels
- reduced level in cellular membranes

3.1. Intake

Usually, HD patients have sufficient daily dietary intake of vitamin E, but sources of vitamin E are limited and mainly represented by vegetable oils. Many other sources are restricted in

Food	Portion size	Vitamin E (milligrams of alpha-tocopherol)	A/ARP/NA in hemodialysis patients	Additional observations
Fats and oils				
Vegetable oil, wheat germ	5 mL (1 tsp)	7	NA	High content of phosphorus
Vegetable oil (sunflower, safflower)	5 mL (1 tsp)	2	A	—
Vegetables and fruits				
Spinach, cooked	½ cup	3–4	ARP	High content of potassium
Tomato sauce	125 ml (½ cup)	2	NA	High content of potassium
Pumpkin, canned	1 cup	3	NA	High content of potassium
Collard greens	1 cup	2	ARP	High content of potassium
Avocado	½ fruit	4	NA	High content of potassium
Mango	1 whole	1.9	NA	High content of potassium
Asparagus, cooked	1 cup	2.2	NA	High content of potassium
Red peppers, raw	1/2	2	ARP	High content of potassium
Grains products				
Cereal, wheat germ	30 g (1/4 cup)		ARP	High content of phosphorus
Meat and alternatives				
Tuna, canned with oil	75 g	2	ARP	High contents of phosphorus
Sardines, canned with oil	75 g	2	ARP	High contents of phosphorus
Herring, cooked	75 g	1.5	ARP	High contents of phosphorus
Egg, cooked	2 large	2.5	ARP	High contents of phosphorus and lipids
Nuts and seeds				
Sunflower seeds	¼ cup	8–10	NA	High contents of phosphorus
Almonds	¼ cup	9	NA	High contents of phosphorus
Peanuts	¼ cup	3	NA	High contents of phosphorus
Peanut butter	2tbsp	3	NA	High contents of phosphorus
Hazelnuts	¼ cup	5	NA	High contents of phosphorus

Table 1. Sources of vitamin E for hemodialysis patients.

these patients because of its increased phosphorus and/or potassium content. **Table 1** displays vitamin E containing foods which are allowed (A), allowed with restricted portions size (ARP), or not allowed (NA) in patients undergoing chronic hemodialysis [4–6].

3.2. Metabolism and clearance

Vitamin E is a lipophilic vitamin. Hemodialysis membranes remove only hydrophilic substances, so that alpha-tocopherol cannot be cleared by hemodialysis. It should be unlikely that vitamin E levels should be low in these patients as long as the intake is adequate, but abnormalities in absorption and metabolism of alpha-tocopherol have been reported.

Tocopherol is metabolized to carboxyethyl-hydroxychromans (CEHC), which are water-soluble compounds excreted by the kidneys. These metabolites accumulate in uremic patients, but as they are water soluble, they might be removed by dialysis membranes also. This could be an explanation for the results of a study from USA that found that even though CEHC levels increased after 30 days of alpha-tocopherol supplementation, they did not increase any more with further treatment [4, 7].

A reduction of vitamin E in cellular membranes has been noted in HD patients, suggesting that a decreased uptake of alpha-tocopherol by different tissues is happening, but the mechanism is not known. Some studies showed a disproportion between plasma tocopherol and lipids, as well as a low level of gamma-tocopherol and CEHC accumulation in patients undergoing chronic hemodialysis.

4. Effects of vitamin E in hemodialysis patients

- antioxidant agent
- antiatherosclerotic
- hypolipidemic
- ameliorates recurrent muscle cramps
- reduces erythropoietin doses

A schematic representation of mechanisms leading to these effects of vitamin E in hemodialysis patients is displayed in **Figure 2**.

4.1. Antioxidant agent

Increased reactive oxygen species (ROS) production was observed in hemodialysis patients, in response to inflammation and extracorporeal membranes. Markers of lipid peroxidation are increased in these patients, while catalase and superoxide dismutase activity are decreased.

5-lipoxygenase branch of the arachidonate cascade is only responsible for membrane peroxidation, oxidative stress, and apoptosis of leucocytes in hemodialysis patients.

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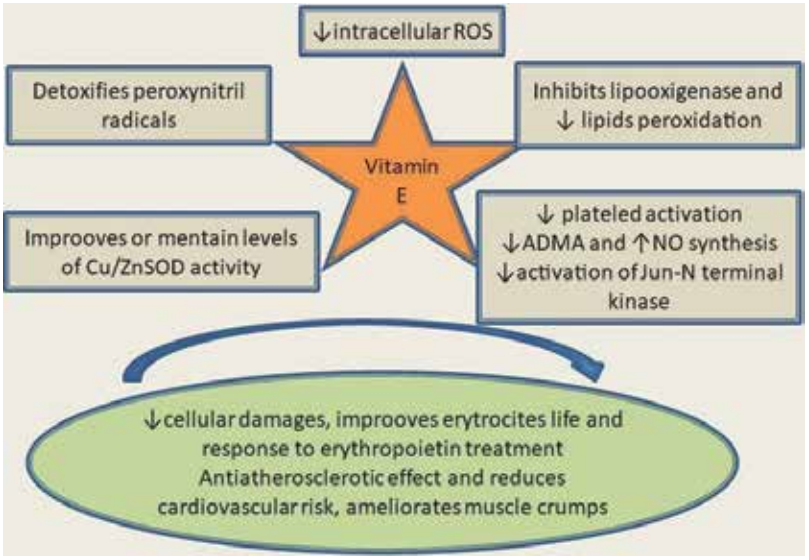

Figure 2. Effects of vitamin E on hemodialysis patients.

Vitamin E might directly inhibit 5-lipoxygenase in peripheral blood monocytes and partially control the lipid peroxidation and oxidative stress [8, 9].

Early studies showed that vitamin E acts as a scavenger for ROS in hemodialysis patients. Other researchers found that gamma-tocopherol is a detoxifier of peroxynitrile radicals. More recent in vitro studies found that vitamin E-coated dialysis membranes reduce intracellular ROS in monocytes and maintain normal activity of Cu/Zn superoxide dismutase [4, 10–12].

Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of endothelial nitric oxide (NO) synthase and it is increased in hemodialysis patients. Vitamin E acts as an inhibitor of ADMA and increases the activity of NO synthase [13, 14].

Pertosa et al. demonstrated that vitamin E-coated dialysis membranes reduce activation of Jun N-terminal kinase [15].

4.2. Antiatherosclerotic effect

Vitamin E acts at the cellular level interfering with reactions implied in the progression of atherosclerosis such as:

- reduces smooth muscle cell proliferation;
- inhibits platelet aggregation and monocyte adhesion induced by superoxide anions;
- reduces lipids peroxidation in monocytes;
- decreases oxidized low-density lipoproteins uptake;
- reduces activation of Jun-N terminal kinase;

- decreases ADMA and increases NO synthase activity and NO synthesis; and
- reduces cytokine production.

All these listed above have been found in small in in vitro or in vivo studies [8–17].

There are two large controlled trials on patients with renal failure, SPACE and HOPE studies, that found either a significant reduction in cardiovascular risk and myocardial infarction in end-stage renal disease patients treated with oral alpha-tocopherol as compared to placebo, or similar effect of vitamin E treatment and ramipril on cardiovascular outcome in patients with mild and moderate renal failure [18, 19].

4.3. Hypolipidemic effect

It was demonstrated that vitamin E reduces lipid peroxidation and it decreases electronegatively charged LDL-subfraction, but if it also reduces the triglycerides or total cholesterol, it is uncertain. Some studies found that short-term high doses of vitamin E had no benefits in a majority of renal patients in regards to their circulating levels of high-density lipoprotein cholesterol [16, 17].

4.4. Ameliorates muscle cramps

Vitamin E-deficient muscle in animals has been shown to provide increased susceptibility to peroxidative damage.

Radical-mediated oxidative damage of skeletal muscle membranes has been implicated in the fatigue process. Moreover, in hemodialysis patients, ROS-mediated damages occur during hemodialysis-induced muscle hypoperfusion and ischemia, as well as because of activation of macrophages and leukocytes passing dialysis membranes. Vitamin E is a major chain-breaking antioxidant that has been shown to reduce contraction-mediated oxidative damage. Vitamin E deficiency would adversely affect muscle contractile function, resulting in a more rapid development of muscular fatigue during exercise [4, 20].

4.5. Erythropoietin doses reduction

Anemia is an important cause of morbidity and mortality in chronic hemodialysis patients. Treatment with erythropoiesis-stimulating agents is influenced by many factors that can induce erythropoietin resistance, such as:

- bleeding
- iron deficiency
- folate and vitamin B12 deficiency
- inflammation
- oxidative stress

An imbalance between oxidant and antioxidant system in hemodialysis patients is well known. Any studies found low levels of superoxide dismutase and decreased activity of

erythrocyte superoxide dismutase in erythrocyte membranes, leading to increased reactive oxygen species production, increased anemia, and resistance to erythropoietin treatment. Some authors found that vitamin E supplementation was followed by increased activity of erythrocyte superoxide dismutase, improving anemia and erythropoietin responsiveness in these patients [4, 10, 21].

5. Vitamin E supplementation in hemodialysis patients

5.1. Oral supplementation

The European Best Practice Guidelines on Renal Nutrition recommend a daily supplement of 400–800 IU for the secondary prevention of cardiovascular events and recurrent muscle cramps [4, 20, 22].

5.2. Vitamin E-coated membranes

Vitamin E-coated membranes were used in hemodialysis to increase membrane biocompatibility and to reduce reactive oxygen species production [23–28].

Effects of vitamin E-coated dialysers [23–28]:

- reduce the levels of advanced glycation end products
- reduce reactive oxygen species
- prevent monocyte activation
- improve the functional capacity of white blood cells population
- reduce lipid peroxidation
- reduce intima-media thickness into carotid artery
- reduce the percentage of dysmorphic red blood cells
- reduce erythropoietin doses

Disadvantages of vitamin E-coated membranes:

- expansive
- not widely available

6. Side effects of vitamin E supplementation

Usually, no side effects have been seen using doses of 400–800 UI/d, even though, in prolonged administration, some of the effects listed below could be possible [20, 22]:

- accumulation of metabolites;

- paradoxical pro-oxidant effects due to the reduction of antioxidant defense system components;
- bleeding, diarrhea, blurry vision, and headache are rare.

In conclusion, vitamin E proved its antioxidant effects in hemodialysis patients, decreasing ROS synthesis and cellular damages, reducing lipid peroxidation, platelet aggregation and limiting atherosclerosis, improving antioxidant defense and ameliorating anemia treatment in hemodialysis patients. It is safe to use doses of vitamin E that should not exceed 1000 Ui/d in selected hemodialysis patients, especially to reduce cardiovascular risk, improve muscle cramps, and reduce required erythropoietin doses.

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Chapter 3

Cytoprotective Effect of 120 Hz Electromagnetic Fields on Early Hepatocarcinogenesis: Experimental and Theoretical Findings

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Paulo Eduardo Ambrosio and
Dany Sanchez Dominguez

Additional information is available at the end of the chapter

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Abstract

Carcinogenesis induced chemically produces mutations affecting standard cells' behavior. An electrophilic attack on DNA result as the primary characteristic. Once xenobiotics are administrated to mammals they suffer a metabolic activation in the liver through cytochrome P450 (CYP450) enzymes, converting them to toxic compounds, generating oxidative stress (OS), and bursting electrophiles near the site of oxidation. CYP450 are electron carrier proteins that generate spin-correlated radical pair (RP) intermediaries. An extremely low-frequency electromagnetic field (ELF-EMF) can modulate the spin-flip conversion between singlet and triplet spin states of the RP populations, modifying the product formation during their metabolization. Experimentally, we induce hepatic cancer chemically; we found that ELF-EMF inhibits both the number and area of preneoplastic lesions by more than 50%. Furthermore, theoretically, we develop a quantum mechanical model based on the RP mechanism (RPM) in the Haberkorn approximation to explain the cytoprotective effects of ELF-EMF. Here, we review the status of the action's mechanism of ELF-EMF on our research on early hepatocarcinogenesis.

Keywords: extremely low-frequency electromagnetic fields, quantum mechanics, radical pair model, hepatocarcinogenesis, enzymatic reactions, cytochrome p450

1. Introduction

Cancer is one of the most burdensome illnesses for humans in the present century, representing a significant public health problem, killing more patients than cardiovascular diseases, and provoking an enormous social impact [1]. The disease gives rise to an enormous economic

cost and human sorrow [2]. In the first instance, all cancers can be prevented. However, 70% of worldwide cancer mortality arises in low-income countries and it is predicted that the number of deaths will grow from 5.5 to 8.9 million by 2030 [3]. In general, researchers expect that there will be 21.7 million new cancer patients and more than 13 million cancer deaths, purely from population growth extrapolation [4]. Notwithstanding this, to develop cancer takes many years because this disease is a syndrome of the adult age. The burden of cancer is increasing due to the growing and aging population and because of unhealthy behaviors, such as smoking, alcohol consumption, unhealthy eating, stress, and physical inactivity [5]. Nevertheless, cancer is a slow and devious killer; almost all cancers might be circumvented through prevention, early detection, and treatment [2]. When cancer has been diagnosed, depending on the kind of cancer, treatment involves surgery, radiotherapy, chemotherapy, and immunotherapy, among others, whose success depends on an assortment of factors. Therefore, the development of successful strategies against cancer can be achieved only through an in-depth understanding of the fundamental biological mechanisms that provoke it. With cancer, we are referring collectively to a considerable number of diseases described by an uncontrolled proliferation of genetically altered cells, generating tumors that can metastasize and invade whole organisms, eventually killing the patient [6]. This means that the best knowledge concerning cancer genesis is needed, focusing on preventive and new tailored forms of treatment. This knowledge will be possible through a multidisciplinary effort, where mathematics and physics collaborate with oncologists, molecular biologists, bioinformatics, and many other disciplines to improve the traditional biological method, enriching it with the implementation of new forms and tools of analyses that permit the acceleration of regulatory barriers in health systems that streamline the implementation of new successful therapeutic strategies [7]. In our research group, we have implemented the use of an ELF-EMF to study hepatocarcinogenesis in its early stages to assess its cytoprotective effect, both experimentally and theoretically. We proposed that it is possible to deter the carcinogenic process induced by chemical carcinogens in the early stages during enzymatic procarcinogen activation by CYP450 through the modulation of charge migration in the electron transfer reactions involved in oxidation [8, 9]. This has been achieved by modulating the magnetic sensitivity of short-lived RP intermediaries produced during the catalytic cycle using ELF-EMF. In this chapter, we review the experimental and theoretical findings found by our research group within the context of the current knowledge on the cytoprotective effect of ELF-EMF in the early stages of OS induced by chemical carcinogenesis (ChemCar). We use the term cytoprotection because of the conferred protection that dissuades or modulates, controls, or deviates processes originated from bioactivation of procarcinogens, which avoids or diminishes damage to cells or its molecular components [8, 9]. On the one hand, we describe and discuss the experimental findings of our group on the effect of daily treatment with 4.5 mT (120 Hz) of ELF-EMF. The early stages of OS in rats with chemically induced hepatocarcinogenesis (ChemIndHep) employed the modified resistant hepatocyte model (MRHM) with Fischer rats 344 [10]. Otherwise, as the molecular mechanisms responsible for this effect are still unclear, we developed a quantum mechanical model based on the RPM and the Haberkorn approximation to explain the experimental effects of ELF-EMF on the free radicals produced in the early stages of ChemIndHep [8]. It is plausible to assume that, through the employment of RPM using ELF-EMF, we modulated the RP intermediates involved in free radical generation (as has been reported for other

reactions [11]). Then, directing them towards lower energetic states in such a way that the activation of oxidative products diminished, and the electrophile damage to cells is reduced [9]. Thanks to this multidisciplinary analysis, we can understand the carcinogenic chemical process based on the behavior of charged particles generated during the enzymatic activation of the procarcinogen, that is, when the DNA still has not yet been damaged in the early stages of carcinogenesis [8, 9]. The results of this work allow us to advertise the basis for the design of tailored therapeutic strategies or clinical applications of ELF-EMF as a coadjutant in the treatment of several diseases related to oxidative damage as occurs in cancer [8, 9]. The importance of the use of ELF-EMF-based therapies is precisely their low cost, safety, effectiveness, and their implication on the homeostasis of the biological systems (BS). We can use them in several treatments, such as chronic ulcer healing [12–14], diabetic foot treatment [15], epilepsy treatment, and vascular permeability in the rat brain [16], among others. In the scientific literature, they have been employed in many fields, and the older applications were in the bone regeneration. However, with the problem that concerns us, we can find many examples. (1) Cells of human colon adenocarcinoma exposed to ELF-EMF of 1.5 mT (1 Hz) during 360 min, diminishing in growth [17, 18]. (2) PC-12 cells exposed to ELF-EMF of 50 Hz at several intensities and durations show a decrease in the proliferation rate and their morphological differentiation [18, 19]. (3) Patients with a mean age of 60 years and with stage IV tumors were enrolled in a pilot study. The patients were exposed for 5 days/week over 4 weeks in two different schedules of exposure: one daily for 20 min (four patients) and one daily for

Table 1. Contents of the chapter.

70 min (seven patients). The results showed that patients do not present side effects. Furthermore, this setup evidenced that humans exposed to ELF-EMF with determinate physical characteristics have a safety profile and with excellent tolerability to the treatment [18, 20]. (4) Patients with advanced hepatocellular carcinoma (HCC), stage I/II, were treated with very low levels of EMF modulated at specific frequencies to HCC. The strategy of stimulation was to administer three daily 60 min outpatient treatments until they arrive at disease progression or death. Most patients reported during the treatment the disappearance or diminishing of pain. Four patients presented with a partial response, and 16 patients had stable disease for more than 3 months. This kind of treatment represents a safe and well-tolerated procedure and also provides evidence of the anticancer effect in this type of patient [18, 21]. We follow the distribution of the syntactic themes expressed in **Table 1** for the development of the chapter's content.

2. Carcinogenesis

Carcinogenesis is a slow process categorized by the acquirement over time of accumulated mutations and chromosomal changes caused by impairment in the genome that leads healthy cells to become deregulated [22]. During cancer development, are subjected monoclonal or polyclonal malignant cells [23] to a microevolutionary process [24] accumulating critical mutations in a crucial group of genes involved in cell division, apoptosis, DNA repair, and in other essential genes that control collective cell behavior. However, after some critical mutations in genes that maintain genetic stability in healthy cells, cancer cells turn into the mutator phenotype [25, 26], initiating a cascade of mutations through the genome that produces genetic heterogeneity in tumors [23]. This process provides cancer cells with selective advantages to colonize the patient's organism evading its natural defenses used to handle any cellular attack. Such benefits of neoplastic cells have been called the hallmarks of cancer and include six features: sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis [27]. Transformed cells, then, instead of behaving altruistically and cooperatively, selfishly ignore any regulating signal and proliferate in an uncontrolled way forming tumors, invading organs and tissues, and spreading throughout the organism, colonizing it.

2.1. ChemCar

ChemCar is a multistep process initiated by the electron attack to nucleophilic tissues or molecules, such as DNA, producing mutations that lead normal cells to become unregulated, proliferate, and then turn malignant [8, 9, 28]. Metabolic activation of procarcinogens produces highly reactive chemical species that yield to the overproduction of reactive oxygen and nitrogen species (ROS, NOS), which cause damage to DNA and other biomolecules [29, 30]. The current mutation theory of carcinogenesis considers it to be a complicated process that broadly consists of three phases or stages: initiation, promotion, and progression (for a review see [31–33]). Chemical carcinogens may act together or in sequence to initiate or promote

carcinogenesis. The initiation stage is characterized by an irreversible dose-dependent genetic change that predisposes normal cells to evolve into a malicious and immortal state [27]. During initiation, cells are induced to proliferate, but not differentiate [33], inheriting mutations and thus producing initiated daughter cells. In the promotion stage, the initiated cells are clonally selected to expand through an increase in cell proliferation or by a decrease in cell death, the so-called apoptosis process [30]. Although promoter stimuli do not interact directly with the DNA, they can be damaged indirectly by OS, and gene expression can be altered by epigenetic mechanisms. Nevertheless, it is considered that promotion is a reversible stage because the elimination of the promoter produces regression in cell proliferation maybe by apoptosis [33]. Lesions in both initiation and promotion stages are yet to be considered as preneoplastic ones or benign neoplasias [32]. However, in the irreversible third state, they are transformed into malignant lesions, i.e., progression. Cell proliferation independent of stimulus characterizes this stage: faster growth, invasion, metastasis, and morphological, biochemical, and metabolic changes in transformed cells [33].

2.2. Carcinogens

There exist a few different mediators that cause cancer, such as biological and physical types, but cancer induced by chemical agents is more frequent [34]. There are a lot of substances or their mixtures identified as carcinogens in the environment, in the diet, in workplaces, in homes, in cosmetics, in the chemicals employed for food production, and even in drugs for human therapeutic use [35]. It has been considered inclusive that cancer could be prevented just by identifying potentially carcinogenic chemicals and eliminating their subsequent human consumption [32]. However, such a practice does not avoid that patients have another kind of cancer. For this reason, search knowledge concerning the chemical and biological mechanisms of cancer development, mainly at very early stages, will contribute to our better understanding of the true nature of this perplexing sickness [8, 9]. Also, it will offer opportunities to generate better strategies to tackle neoplasia or to interrupt the process [32]. It is known that the earlier the detection and treatment of cancer, the better the probabilities of control, prevention, and cure because treatment will be more effective when cancer cells have not yet invaded nearby tissues or metastasized through the body [8, 9, 36]. Furthermore, it is best to interrupt carcinogenesis as soon as possible, maybe in the preneoplastic stages or, even more, at very early stages when carcinogens or by-products of their metabolism have not yet confronted the DNA. In this manner, it could be possible to prevent the progression of the sickness to the invasive terminal stage. The scenario to be considered to identify what mechanisms are needed to be stabilized, arrested, or even reversed [37] should be based on the correct approach to the chemoprevention field in a multidisciplinary study. Cancer research can involve physics and mathematics using physical, natural, synthetic, or biological agents, and mathematical tools of analyses and simulation in computers to reverse, suppress, or prevent either the initial phases of carcinogenesis or the progression of the premalignant cells to invasive sickness [38]. We use ELF-EMF together with quantum mechanics models to assess cancer. Chemical carcinogens are classified into two groups: (1) direct-acting or primary carcinogens and (2) indirect-acting or procarcinogens [8, 9, 39]. Direct-acting carcinogens are compounds sufficiently reactive and electrophilic that they interact directly with DNA forming adducts and chromosome breakage,

fusion, deletion, missegregation, or nondisjunction that lead to genomic damage. Conversely, procarcinogens do not interact directly with the genome until they are metabolically activated, producing genotoxic electrophilic metabolites or ROS. We consider that an essential critical point is the process of activation of chemical procarcinogens in which the proper enzymatic reaction of the substrate produces electrophiles and ROS [8, 9]. CYP450 is responsible for these enzymatic reactions. CYP450 is a family of proteins that share common mechanisms of activation of procarcinogens that result in OS [30, 40]. A particular event in ChemCar is the very origin of reactions in cascades, intermediates, and products, which constitute the insult to cells after procarcinogens have been incorporated into the organism [8, 9]. Thus, downregulation of ROS and NOS could contribute to the prevention of cancer initiation [38].

2.3. Metabolization

Usually, drug metabolism is the process of biochemical transformation using drug metabolism enzymes (DME) of xenobiotics, i.e., exogenous compounds introduced into organisms. The organs and tissues of animals have a diversity of DME that protects the body against both potential harmful substances from the environment (xenobiotics) and certain substances produced by the organism itself (endobiotics) [41]. Detoxification reactions comprise three phases and enzymes: phases I and II consist of metabolization reactions and enzymes, and phase III consists of transporters involved in efflux mechanisms [42]. Enzymes of phase I participate in reactions of conversion, mainly involving oxidation, reduction, and hydrolysis. These are classified as oxidoreductases (such as CYP450 monooxygenases, flavine monooxygenases, cyclooxygenases, and alcohol dehydrogenase) and hydrolases [33, 43]. Conversely, enzymes of phase II participate in reactions of conjugation and inactivation of chemical carcinogens and include transferases. Altogether, enzymes of both phases occurring independently, sequentially, or simultaneously transform xenobiotics into polar water-soluble and excretable metabolites [41]. In mammals, the metabolism of exogenous chemicals is carried out mainly in the liver, the primary site in which CYP450 enzymes are present [44]. Although procarcinogens tend to be chemically stable, they are metabolized in the liver by CYP450, a phase I enzyme, which detoxifies typically xenobiotic compounds. The activity of detoxifying enzymes in phase I is critical for carcinogenic activation of xenobiotics, while the activity of enzymes of phase II is essential for xenobiotic neutralization [45].

2.4. The catalytic cycle of CYP450

During the catalytic cycle of procarcinogen activation carried on by CYP450, short-lived spin-correlated RP intermediates are produced, which can either recombine or continue the catalytic process. The cycle initiates at resting state with the enzyme in the ferric form where a water molecule is implemented as the sixth ligand to iron in the active site. The ferric state of the resting enzyme has its five valence electrons occupying its orbitals, equilibrating the low-spin and high-spin state [46]. CYP450 at the beginning has ferric iron predominantly in the low-spin state. The substrate induces conformational changes displacing the water molecule from the distal axial coordinate position of the heme iron. This fact results in iron displacement from the porphyrin plane, which makes the heme a better electron sink and triggers electron

transference [46], changing the oxidation/reduction potential in the spin state of the heme iron from the low-spin form towards the high-spin form, which is a better electron acceptor. Thus, the electron donor, which could be an iron/sulfur protein or a flavoprotein, reduces the high-spin iron(III) heme to the high-spin iron(II) state. This intermediate compound has a singlet ground state and is a resonating mixture of the ferrous and ferric forms. This conjugation requires that the dioxygen will be in a singlet state so that the empty orbital of the oxygen can mix with the second occupied orbital of the iron [46]. It is transferred in the oxygen from the activated CYP450, that is, compound I, to the heteroatom of the substrate. It has been described in the general catalytic mechanism as an odd-electron process, which involves the transfer of one electron or hydrogen atom to generate an intermediate complex that collapses by recombination [47].

3. Experimental findings

3.1. Hepatocarcinogenesis

Hepatocellular carcinoma (HCC) is one of the public health enemies in low-income countries like Mexico and Brazil. It represents the fifth cause of death in the economic stage of man and ninth in women [48]. Therefore, it is important that HCC is properly understood. HCC is induced by several factors: environmental, infectious, nutrimental, metabolic, and endocrine. Certain factors such as chronic infection with hepatitis B and C, aflatoxin exposure, excessive consumption of alcohol, tobacco, and polysaturated meat consumption can also increase risk [49]. In fact, HCC is associated with liver cirrhosis and chronic hepatitis. Hepatocarcinogenesis is a complex multifactorial phenomenon that appears with loss of heterozygosity, somatic mutation, methylation, and functional inactivation [49]. The disease has a poor prognosis despite the pathophysiological advances and treatments. Chronic liver disease has an initiation point with intrahepatic inflammation promoting the dysregulation of cellular signaling pathways, triggering cell proliferation, and expanding malignant cells [50]. During this stage OS and metabolic disorders appear.

3.2. Nrf2 transcriptional factor + ELF-EMF

The Nrf2 (nuclear factor erythroid 2-related factor 2) transcription factor offers essential protection to cells against OS, binding antioxidant response elements and detoxifying enzymes such as glutathione *S*-transferase A2 and NADPH quinone oxidoreductase [51, 52]. In normal conditions, Nrf2 is found in cytoskeletal protein Keap1 (Kelch-like ECH-associated protein 1). However, when ROS and electrophiles are present, it is dissociated from Keap1, translocating Nrf2 to the nucleus, activating cytoprotective genes that participate in the electrophile conjugation and the excretion of xenobiotics. Since Nrf2 activates phase I and II enzymes, it can be considered as a target for cancer chemoprotection [53]. However, in recent studies, the beneficial antioxidant activity of Nrf2 has been extended to protect cancer cells, since excessive Nrf2 activity provokes mutations in NFE2L2 or Keap1, avoiding chemotherapy efficiency [52, 54, 55]. Another study gave evidence of Nrf2

activation in cancer [56]. Despite the enormous benefits and therapeutic chances offered by the use of Nrf2, however, the regulatory mechanisms involved at the molecular level are still not completely clear. Furthermore, ELF-EMF induces the activation of the antioxidant pathway of Nrf2, associated with the protective effect induced by the administration of 3-nitropropionic acid, which causes neurotoxicity [57], showing that ELF-EMF mitigates oxidative damage [58, 59]. For this reason, it is crucial to implement other useful tools to gain knowledge concerning carcinogenesis.

3.3. Experimental setup

We induce experimental hepatocarcinogenesis through the use of diethylnitrosamine (DEN) when it is hydroxylated by the CYP450 isozymes in the liver, employing an alkylation mechanism to bioactivate it, and reacting with DNA, causing ethylation in their bases, which are called DNA adducts. When those interrupt the base pairing, they provoke mutations and activation of proto-oncogenes such as ras and inhibition tumor suppressor genes such as p53, generating HCC [60]. In our experimental setup in Ref. [10], where MRHM was implemented to generate ChemIndHep, it was found that provoking the rapid proliferation of altered hepatocytes formed preneoplastic lesions in the rat liver to assess the effects of ELF-EMF on hepatocarcinogenesis. The results indicate that applying periodically an ELF-EMF was possible for achieving the modulation of the magnetic-sensitive short-lived RP intermediaries produced during the catalytic cycle. Such daily treatment with ELF-EMF (4.5 mT (120 Hz)) inhibits more than 50% of the number and area of preneoplastic lesions in rats through reduction of cell proliferation and without altering the apoptosis process. The general idea is straightforward: modulate, applying ELF-EMF, the potentially harmful products yield of the reaction by competitive kinetics of RP selective reactions [8], which give the role of a molecular motor to the enzymatic protein. Whose provision of catalyzing electrons to the reaction, is executed when through the substrate of CYP450 are metabolized the xenobiotics that participate in the ChemIndHep [9]. During the implementation of MRHM, the rats were administered a single necrotic dose of DEN (200 mg/kg b.w., i.p.). Seven days later, over 3 consecutive days, the rats were administered 2-acetylaminofluorene (20 mg/kg b.w., orally), after which they were subjected to a two-thirds partial hepatectomy. We employed three groups of six rats (normal control group (NC), sham-exposure group (CT), 120 Hz ELF-EMF group (CTF)) and the biochemical and molecular evaluations were performed under blind conditions.

3.4. Experimental results

DNA fragmentation through a calorimetric TUNEL system kit found that daily treatment with ELF-EMF did not induce apoptosis in the altered hepatocytes or caspase-3 activation, showing that ELF-EMF interferes with altered cell cycle continuity and DNA synthesis induced by ChemIndHep. This analysis also was used to detect the endogenous peroxidase activity, showing an essential diminishing in the numbers and areas of glutathione-*S*-transferase-positive (GST-P) liver foci and preneoplastic liver lesions in rats (**Figure 1**). To evaluate the

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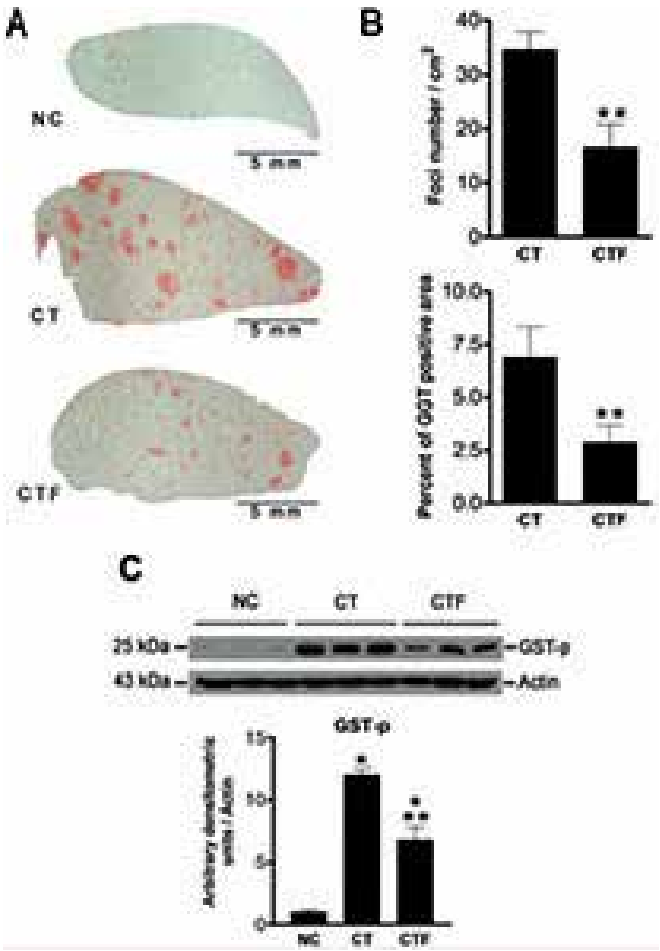

Figure 1. Effect of ELF-EMF exposure on GGT-positive lesions and GST-P expression. (A) Representative liver sections of the NC group, CT group, and CTF group. Scale bars, 5 mm. (B) Quantification of the foci number/cm^2 and percent of the GGT-positive area. (C) Western blot analysis for GST-P expression. GST-P was normalized with actin expression used as the loading control. The expression of NC was adjusted to one in the densitometric unit scales. Statistically different from *NC and **CT, $P < 0.05$. Data are expressed as the mean ± SEM; $n = 6$ for each group (of reference [10]).

effect of ELF-EMF on cell proliferation, immunohistochemical analysis of protein expression of the cell cycle such as PCN, Ki-67, and cyclin D1 were also performed. PCNA participates in replication and DNA reparation, a fundamental process for the cell cycle. Otherwise, Ki-67 is a specific replication marker of the cell cycle, which does not include the G_0 phase. Summarizing, the daily application of 120 Hz ELF-EMF affects the ChemIndHep process participating in the reduction of protein expression of PCNA, Ki-67, and cyclin D1. Their participation does not induce apoptosis; however, it regulates cellular homeostasis inhibiting the development of preneoplastic lesions.

4. Theoretical findings

Currently, it is known that both permanent magnetic field (MF) and ELF-EMF interact with BS at all levels [61]. Nonetheless, there still does not exist precise molecular mechanisms for which ELF-EMF can support their therapeutic use, although much research has been performed and reported of several effects on BS [9]. One of the most common reports is that an external MF can considerably affect the rates of chemical reactions (ChemReac) in BS. Involving free radicals affects the probability of transition between the singlet (S) and the triplet spin state (T), $S \longleftrightarrow T$, generally in the RP [62, 63]. Hyperfine interaction (HypInt) is responsible for control of the spin-flip conversion $S \longleftrightarrow T$ spin states of the RP modifying it by the application of an external MF [9]. Physically, there exist four orders of magnitude between molecules interacting with a geomagnetic field of 50 μT with respect to the vibrational, thermal energy $K_B T$ compared to the strength of a chemical bond, which is 10–100 times smaller [9, 64, 65]. Therefore, adaptive mechanisms to deal with such energies [66] could not be developed, except the geomagnetic field [67], which today as a way of orientation is used [64]. As a result, organisms, considered as complex electrochemical systems, interact in a very complex and subtle way with EMF. Nevertheless, several studies have demonstrated the influence of EMF on BS and diseases, mainly by intensifying the effects of other physical and chemical stressors [61]. In fact, it is possible to verify that static MF [68] or pulsating MF [69] can affect chemical systems and free-radical reactions in BS [70]. For enzymatic reactions, in 1994 Harkins and Grissom reported the MF effect on coenzyme B_{12}-dependent ethanolamine ammonia lyase in vitro activity as evidence of the RPM [9, 71]. When it is used at a low frequency, the effects of weak MF on BS were revised by Liboff [72] using ideas concerning cyclotron frequency resonances. Using vibrational modes by Lednev [73], and the Zeeman levels by Blanchard et al. [74], hyperfine interactions with a one-proton model were treated by Haberkorn and Michel-Beyerle [75], among others [9]. Therefore, the absence of a convincing molecular mechanism is the reason why there exists a significant unconformity in the research community with respect to the existence of certain MF or ELF-EMF effects (MFE or ELF-EMFE) on BS and ChemReac. In this respect, the RP intermediaries simultaneously created, with a spin state correlated is the key. These MF-dependent particles are controlled by a weak MF $(\leq K_B T)$ thanks to their spin correlation, which is far away from the thermal equilibrium. Thus, the RPM seems like the most plausible way to investigate the MFE or ELF-EMFE on the reactivity of ChemReac [64, 65] in BS. A notable example of MF on BS is the photosynthetic reaction center of proteins in which light absorption permits RP formation due to the electron transfer steps [64, 65]. An exceptional case is a report about MFE on the enzymatic synthesis of adenosine triphosphate in cells to control enzymatic deoxynucleic (DNA) synthesis in cell proliferation by Buchachenko [76]. After all, phosphorylation is of vital importance for the function of BS. There exist three sources of irreproducibility of MFE: the presence of paramagnetic metal ions [77], the existence of catalyzing metal ions [76], and kinetics and RP spin dynamics [9, 78]. Besides, there is extensive fundamental and clinical accumulated evidence regarding the effectiveness of ELF-EMF in therapeutics and clinical benefits, and in the significant modulation of molecular and cellular function [61].

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4.1. Fundamental facts

Facing our limited knowledge and using the available information, we reported the cytoprotective effect of 120 Hz ELF-EMF on early ChemIndCar during the enzymatic procarcinogen activation of CYP450 by quantum measurements in Refs. [9, 79]. We proposed that when CYP450 metabolizes the xenobiotics used in the experimental setup to ChemIndHep [10], the enzymatic proteins act as a molecular motor providing a catalyzing electron that interacts with the RP formed during the OS generated when the substrate of the enzyme is oxygenated [9]. Since metabolization is carried on in the liver, the hepatocytes are in contact with the enzymatic protein as in a thermal bath and with a Gibbsian distribution, interacting with the RP as a harmonic oscillator [9]. Employing quantum measurements concepts, we argue the way in which the MF modulates the singlet spin population to diminish the preneoplastic lesion observed during ChemIndHep. The completely formed system between RP and electronic configuration of hepatocytes interacting through the HypInt alters the quantum spin state removing the spin prohibition and giving rise to the appearance of new reaction products. These products in our case result in spin selectivity plus HypInt, affecting the magnetic properties, which impact on the so-called initiated hepatocytes that later become the preneoplastic lesion to study.

We explained that on the formation of the OS was involved through the administration of xenobiotics in the MRHM, an electron transfer, and the MF modulates those in the current Haberkorn approach [9, 79, 80]. The allowed electrophilic reactions that appear in the enzymatic reactions do not require a change of spin because the spin total is zero. Those spin-forbidden reactions, involving paramagnetic participants, can combine their spins freely in any electronic configuration, but it does not mean that all configurations have a chemical reaction [9, 79]. The electron spin gives origin to the MFE, the magnetic isotope effect, and induces nuclear chemical polarization. We use the fact that according to angular momentum conservation, which is a fundamental and universal principle, all ChemReac are spin selective. This means that only those ChemReac that satisfied such a rule are allowed in the product formation. Magnetic interaction is the masterpiece that controls and accelerates the ChemReac. Nevertheless, it is many orders of magnitude less than coulomb energy. However, it has the responsibility of changing the electron spin state through the interchange energy of the channels of spin allowed and prohibited, controlling chemical reactivity [81]. The MFE act over the enzymatic DNA synthesis killing cancer cells [76]. We use the RPM because it controls life at the molecular level [9, 82–84]. We use the fact that a combination of weak static and pulsating EMF can affect radical concentration in a ChemReac [85], modifying the population of nuclear and electron spin states, their energy levels, and the RP alignment of their magnetic moments. Such changes can modify the BS [9]. Also, we use the experimental findings concerning the significant modulation of catalase, CYP450, and inducible nitric oxide synthase activity in myelogenous leukemia cells [86] to reaffirm our idea with respect to the effect of ELF-EMF on the enzymological system. A more critical step is the activation of xenobiotics whose interaction produces OS in the form of electrophiles and ROS [30, 40]. The main protagonist in our approach are the so-called RP, short-lived intermediates that participate in almost all reactions in solution in a correlated way. The RP can recombine or participate in other ChemReac. They

are responsible for a few phenomena such as chemical polarization of electrons and nuclei, and the influence of static and pulsating MF [9, 79]. An RP can decay by recombination, or pull apart the radical by diffusion, or react with other radicals. One of the properties of RP is that recombination probability depends on spin multiplicity, and it varies during RP lifetime. Such variations, as an interesting detail, are manifested as dynamic quantum oscillations, the so-called quantum beats between (S, T) spin states of the RP. The quantum beats modulate the probability of appearance of some reaction channels of the RP that at the time affect the MFE. By studying these quantum beats, one can reveal valuable information concerning the structure, reaction, molecular, and spin dynamics of RP [9, 63, 79, 87]. The RP spin correlation is formed in the coherent state, which oscillates between the S and T spin state, an oscillation that depends on the spin Hamiltonian operator parameters (see references [8, 9] for details), in particular that of the HypInt. The period of the oscillation on organic radicals is in the range of nanoseconds, making RP recombination a plausible test that suggests that small EMF affects BS [9, 88].

4.2. The model

ChemReac can be seen as a physical process involving a set of regrouping atoms with the rearrangement of electronic shells of reacting participants, giving place to the generation of new molecular structures called reaction products. The new ways of controlling ChemReac have their basis on the selectivity of spin, a process involving the spins of molecules, electrons, and nuclei of all participants. For this reason, the rate of spin-selective processes is dependent on MF, which alters the spins of the participants, changing partially or wholly the spin selectivity [9, 89, 90]. Thus, to reveal the interaction to explain the cytoprotective effect of ELF-EMF in CYP450, we must define the conditions where the quantum measurement is performed. The first condition is that all quantum states participating in the hepatocytes-RP system in the enzymatic reaction are singlets, because of their high reactivity. The second condition is during the enzymatic procarcinogen activation of CYP450 when the xenobiotics are metabolized, in which appear the RP when is generated the OS. The RP intermediaries are produced in this step, and they are responsible for the insult to hepatocytes, which become the future preneoplastic lesions after to finish the ChemIndHep. The third condition is daily MF stimulation during all ChemIndHep. Nonetheless, the spin evolution of RP is driven by the MF through the HypInt and their reactivity is controlled by spin dynamics, converting non-reactive triplets into reactive singlets through quantum measurement. We showed the way in which the MF modulates the charges in migration evaluating the recombination probability to exemplify. In this respect, when the RP interacts with another electrons' spin, this interaction acts as a catalyst, increasing the recombination probability and accelerating the $S \longleftrightarrow T$ interconversion [9, 90, 91].

4.2.1. Haberkorn approach

To study the hepatocytes-RP system, we use the information of all parameters employed in the evaluation of MFE, the recombination yield, and the singlet population, which are all included in the Haberkorn approach; this is the most common theory used for spin dynamics studies.

This approach is obtained using the spin density matrix in the framework of the Liouville–von Neumann equation involving the rate at which singlets disappear, called k_S; it is involved in an unnormalized wave function [9, 80] $\|\psi>= c_S e^{-k_S t/2}\|S> + c_T\|T>$. Here the amplitudes for the singlet disappear at the rate $-k_S/2$, provoked by the interaction between RP and a third electron for the electron configuration of the hepatocytes (see reference [9] for details). We have written the evolution of the standard density matrix as [9]:

$$\hat{\rho}_{\text{in}} = \begin{pmatrix} C_S C_S^* e^{-k_S t} & C_S C_T^* e^{-k_S t/2} \\ C_T C_S^* e^{-k_S t/2} & C_T C_T^* \end{pmatrix} \equiv \hat{\rho}_0 \rightarrow \hat{\rho} = \begin{pmatrix} C_S C_S^* \left[1 - e^{-k_S t}\right] & \tilde{\varnothing} \\ \tilde{\varnothing}^{\dagger} & \hat{\rho}_0 \end{pmatrix}. \tag{1}$$

Moreover, to satisfy the unicity of the trace in the density matrix, we include the third electron. Obtaining the so-called reaction products, $\hat{\rho}$, according to the rule of conservation of the number of entities participating, i.e., for the generation of some product population, $\tilde{\varnothing} = (0\ 0)$ is a null vector [9, 79].

4.2.2. Quantum measurements

When we have a ChemReac with only a singlet spin state as in our case, we can consider it as a quantum measurement [80]; the amplitudes for the singlet disappear at the rate of $-k_S/2$ provoked by the interaction of the third spin electron that can be studied. During their evolution, the RP can change their spin multiplicity. Through the use of electron spin resonance spectroscopy (ESR) studies, such spin changes are simply called beats, meaning dynamical quantum oscillation between the $\|S>$ and $\|T>$ spin states of the RP. Using them, we study the behavior of the spin dynamics of RP. A crucial issue here is that RP appears in the coherent state, which permits the oscillations between $\|S>$ and $\|T>$ spin states of the RP, commanded by HypInt. Measured at a quantum level, these beats represent the manifestation of the RP in ESR studies. Tacitly, the beats correspond to $S \leftrightarrow T$ spin-flip transitions generated by HypInt. The behavior of an unpaired electron under MF, or without MF, determines the influence of HypInt so we can measure the MFE. Eq. (1) expresses how singlet disappears at the desired rate k_S. Nevertheless, the off-diagonal terms represent the coherent superposition decaying at a rate of $k_S/2$. This expresses the motion equation for the density matrix with $\hat{H}_{int}$ as the Hamiltonian interaction operator as (see reference [9], for details):

$$\frac{\mathrm{d}\hat{\rho}}{\mathrm{dt}} = -i\left[\hat{H}_{int}, \hat{\rho}\right] - \frac{1}{2}k_S\left(\hat{\rho}\hat{Q}_S + \hat{Q}_S\hat{\rho}\right), \tag{2}$$

where $\hat{Q}_S = \|S><S\|$ is the projection operator for the singlet state. The yield of recombination calculated from the singlet state of the RP can be evaluated by [9]:

$$\Phi_S = k_S \int_0^{\infty} \mathrm{Tr}\left[\hat{Q}_S\hat{\rho}(t)\right]\mathrm{dt}, \tag{3}$$

In fact, with Eq. (3), we evaluate the effect of MF on the yields of the diamagnetic products involved, and in those RP that do not participate in the recombination process. Furthermore, in

the exponential approach, the Φ_S represents the effect of all reencounter times for the reencounter probability of a diffusive geminate RP when it describes the time evolution after their formation, and $\tau^{-1} = k_S$ is the average reencounter time when $k_S = k_T$ [9]. We use as an initial condition the fact that the population is born in a singlet state $\|\Psi(t=0) >= \|S >$. The evolution time wave function reads $\|\Psi(t) >= \sum_n A_{P_n}(t)\|P_n > + A_S(t)\|S > + A_{T_0}(t)\|T_0 >$. In this sense, the quantum measurements [92] give us the formation of products and then the effect of singlets in the hepatocytes with an intensity $\|A_S(t)\|^2$ [9, 79]. With use of the spin-based quantum mechanical model, we perform the calculation of singlet spin population and determine the MFE, obtaining a result of 61% compared with the experimental findings of 56% and 58%. Evaluating the quantum yield for the RP intermediaries in the substrate-product system of the CYP450, it is interesting to illustrate the cytoprotective mechanism, which consists of the diminution of the singlet population, responsible for diminishing the number of initiated cells, and, therefore, the preneoplastic lesion formation. To study the spin population behavior of the system, we diagonalize the interaction spin Hamiltonian in the superstate representation, applying the Lanczos method [9]. Beyond the mathematical model, the biology of the problem concerns the action of the EMF on RP affecting the hepatocytes during the enzymatic procarcinogen activation of the CYP450, precisely modulating the charges that are in migration during the electron transfer reactions generated by the interaction of the CYP450 in their substrate-producing electrophilic species and ROS. The intermediaries generated during this process are the source of the first insult to hepatocytes on their way to becoming preneoplastic lesions in the ChemIndHep protocol. We used three assumptions: (1) the ChemReac are spin selective, (2) ChemReac are nuclear spin selective, and (3) ChemReac are selective with the spin of the electron. Under such circumstances, only the reactions with singlets favored the formation of standard molecules. The reactions with triplet RP are forbidden. Under this outline, the spin of the electron controls the generation of the magnetic spin effects [9]. One of the keys of the model is to consider the role of the enzymatic protein as a molecular motor, catalyzing electrons to the reaction, where the RP is generated into the substrate of CYP450 when it is metabolizing the xenobiotics used in the MHRM. The hepatocytes are in contact with the RP in the liver as in a thermal bath, and we assume a Gibbsian distribution, interacting harmonically with them. This strategy includes the very tough dissipation problem, whose quantization process involves some difficulties. To do this, we employed the Caldera–Legget model [93, 94], which explicitly includes it. We use the path integral method in the Feynman–Vernon functional approach to describe the time evolution of the spin population of the system. Also, we employ the influence-functional technique to incorporate the Brownian motion at any temperature. Thus, our system can be considered close and we can apply the traditional quantization method [93]. We consider conservation of energy, and to give an exact treatment of the quantum dissipation dynamics, we use the hierarchical equation of motion, which is more tractable from a numerical point of view. We use for simplicity, $H_p = \omega_{osc}\hat{b}^{\dagger}\hat{b}$ modeling the ChemRec and consider the thermal bath as a set of harmonic oscillators with equally spaced energy levels at the frequency ω_{osc}. This expresses the complete system to study as $\hat{H}_{Sb} = \sum_{\alpha\mu}\left(\hat{a}_{\mu}^{+}\hat{F}_{\alpha\mu}^{-} + \hat{F}_{\alpha\mu}^{+}\,\hat{a}_{\mu}^{-}\right)$, representing $\hat{a}_{\mu}^{+} \equiv \hat{a}_{\mu}^{\dagger}$ $\left(\hat{a}_{\mu}^{-} \equiv \hat{a}_{\mu}\right)$, the creation (annihilation) operator of the electron in some specified spin-orbit state. Moreover,

the bath operators $\hat{F}^{-}_{\alpha\mu} = \sum_k t_{\alpha\mu k}\,\hat{a}_{\alpha k} = \left(\hat{F}_{\alpha\mu}\right)^{\dagger}$, whose influence is characterized by bath spectral density functions $J_{\alpha\mu\nu}(\omega) = \pi\sum_k t_{\alpha\mu k} t^{*}_{\alpha\nu k}\delta(\omega - \omega_{osc})$. In our approach such terms are expressed by the reaction operator $\hat{R}_S = \alpha\hat{b}^{\dagger}\hat{N}_{-} + \alpha^{*}\hat{b}\hat{N}_{+}$, representing the spin-selective recombination of the singlet state. To evaluate the recombination process, which is responsible for the time evolution (see details in Ref. [9]), we express the Liouville equation (Eq. 2) in the quantum interaction representation, involving all terms of the system [9] $\frac{d\hat{\rho}_c(t)}{dt} = -i\left[\hat{H}_T + \hat{H}_P + \hat{R}_S, \hat{\rho}_c(t)\right]$, where $\hat{\rho}_c(t) = \hat{U}_0(t,t_0)\hat{\rho}_c(t)\hat{U}_0^{\dagger}$, $\hat{H}_0 = \hat{H}_T + \hat{H}_P$, and $\hat{U}_0(t,t_0) = e^{-i\hat{H}_0(t-t_0)}$ is the evolution operator of the system. Formally, we evaluate all operator quantities involved in the interaction representation. Once we calculate the thermal bath's degree of freedom contributions and apply the detailed balance principle, we arrive at the solution to the Haberkorn approach, $\rho_{SS}(t) = \rho_{SS}(0)e^{-k_S t}$, $\rho_{T_0T_0}(t) = \rho_{T_0T_0}(0)$, $\rho_{ST_0}(t) = \rho_{ST_0}(0)e^{-\frac{k_S}{2}t}$, $\rho_{T_0S}(t) = \rho_{T_0S}(0)e^{-\frac{k_S}{2}t}$, $\rho_{PP}(t) = \rho_{SS}(0)\left(1 - e^{-k_S t}\right)$.

From the last equation, we can present the form in which products are formed. We suppose that in the initial process at $t = 0$, $\rho_{PP}(0) = 0$, there are no damaged hepatocytes. The behavior of the generation of damaged hepatocytes will depend on the initial singlet spin population $\rho_{SS}(0)$, which means that the medium absorbs all that is produced by the enzymatic reaction $\rho_{PP}(t) = -[\rho_{SS}(t) - \rho_{SS}(0)]$ [9].

4.2.3. Results on hepatocytes

We described the effect through the following dynamical mapping [9, 79] $\|S> \|0>_{\mathrm{Hep}} \rightarrow \eta_1\|P> \|\sigma>_{\mathrm{Hep}} + \eta_2\|S> \|0>_{\mathrm{Hep}}$, $\|T_0> \|0>_{\mathrm{Hep}} \rightarrow \|T_0> \|0>_{\mathrm{Hep}}$. When we normalize it, measurement of the generation of some reaction product on the hepatocytes $\|P>$ is represented according to [9, 79]:

$$\hat{\rho}(0) = \begin{pmatrix} \|A_S\|^2 & A_S A^{*}_{T_0} \\ A_{T_0}A^{*}_S & \|A_{T_0}\|^2 \end{pmatrix} \rightarrow \hat{\rho}_n = \beta\begin{pmatrix} \|\eta_1\|^2\|A_S\|^2 & \eta_2 A_S A^{*}_{T_0} \\ \eta^{*}_2 A_{T_0}A^{*}_S & \|A_{T_0}\|^2 \end{pmatrix}, \beta = \frac{1}{1 - \|\eta_1 A_S\|^2}. \quad (4)$$

This fact means that the hepatocytes measure the spin nature of the RP that is participating. The spin state of the hepatocyte changes according to the spin nature of the RP that is interacting. During the measurement process, the S and T_0 components do not change, but the hepatocytes change their spin states according to the RP spin character following the dynamical mapping [9, 79] $\|S> \|0>_{\mathrm{Hep}} \rightarrow \beta_1\|S> \|\sigma>_{\mathrm{Hep}} + \beta_2\|S> \|0>_{\mathrm{Hep}}$, $\|T_0> \|0>_{\mathrm{Hep}} \rightarrow \beta_3\|T_0> \|\chi>_{\mathrm{Hep}} + \beta_4\|T_0> \|0>_{\mathrm{Hep}}$, instituted by the interaction [9, 79]:

$$\begin{aligned}\hat{H}_{in} = &\left\{\eta_S\|S><S\|\right\}\otimes\left[\|\sigma>_{\mathrm{Hep}}<0\| + \|0>_{\mathrm{Hep}}<\sigma\|\right] + \left\{\eta_{T_0}\|T_0><T_0\|\right\} \\ &\otimes\left[\|\chi>_{\mathrm{Hep}}<0\| + \|0>_{\mathrm{Hep}}<\chi\|\right]\end{aligned} \quad (5)$$

where η_S and η_{T_0} give us the strength of the interaction of the hepatocytes with the RP spin character, appearing in the new term, $\beta_3\|T_0> \|\chi>_{\mathrm{Hep}}$, without $\|P>$ states. In this case, the probabilities $\|\beta_1\|^2$ or $\|\beta_3\|^2$ express the appearance of $\|S>$ or $\|T_0>$ spin states, represented by

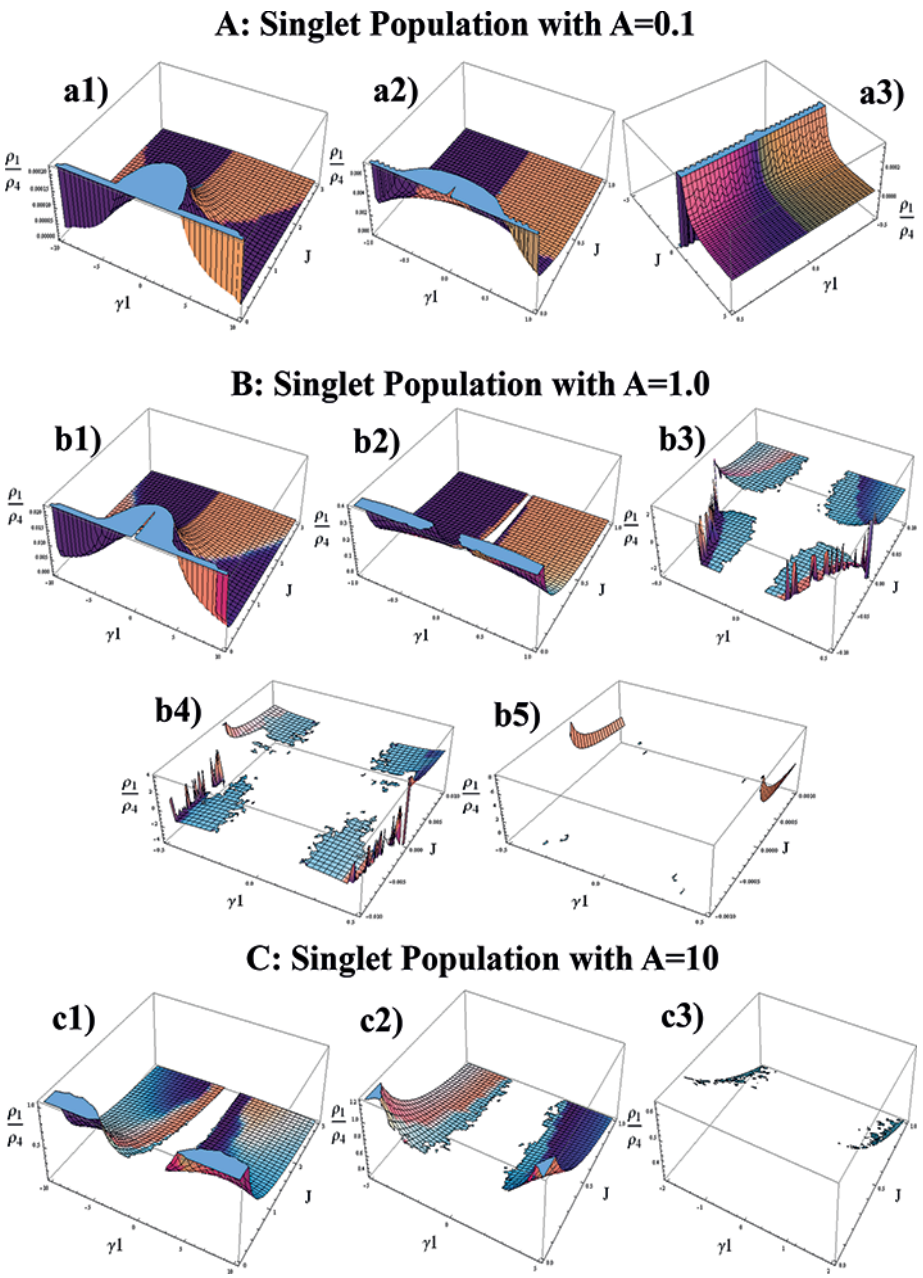

Figure 2. (a) Singlet population normalized with ρ_4 for A (=0.1): With the range of values of (γ_1,J) (a1) [(−10,10), (0,3)], (a2) [(−1,1), (0,1)], (a3) [(−0.5,0.5), (−5,5)]. (b) Singlet population normalized with ρ_4 for A (=1): With the range of values of (γ_1,J) (b1) [(−10,10), (0,3)], (b2) [(−1,1), (0,1)], (b3) [(−0.5,0.5), (−0.1,0.1)], (b4) [(−0,5,0.5), (−0.01,0.01)], (b5) [(−0.5,0.5), (−0.001,0.001)]. (c) Singlet population normalized with ρ_4 for A (=10): With the range of values of (γ_1,J) (c1) [(−10,10), (0,3)], (c2) [(−5,5), (0,1)], (c3) [(−2,2), (0,1)] (from reference [9]).

$\|\sigma>$ and $\|\chi>$ [9]. They measure the fraction of singlets transformed into a reaction product or transformed cell. It is precisely this kind of product formation that changes the electronic configuration of the hepatocytes, as we claim. The result is evident from **Figure 2**, where we note that by increasing the hyperfine coupling constant, A, the singlet population is diminished. Thus, once the hepatocytes interact during δt with the CYP450, they change their spin states through the application of the selective spin operator. For each time interval, δt will apply some dynamical mapping to each new healthy hepatocyte in the tissue $\|0>_{\text{en}}$, incorporating them into the enzymatic reaction that provides catalyzing electrons during the metabolization of xenobiotics [9], $\|\Psi(\delta t)>_{\text{s en}} = \mathbb{U}(\delta t)\|\Psi(\delta t)>_0 = e^{-i\hat{H}_{in}\delta t}\|\phi_0>_{\text{s}}\|0>_{\text{en}}$. Also, neglecting the memory effects due to the previous results with other singlets, which are diminishing as is evidenced by the quantum measurement $\|\beta_1\|^2 = k_S\delta t$, changes the electronic configuration of the hepatocytes when the RP is converted in a reaction product by the recombination kinetics [9, 79].

5. Conclusions

We studied the ChemIndHep experimentally through the use of MRHM. We found that the periodical application with ELF-EMF (4.5 mT (120 HZ)) inhibits by more than 50% the number and area of GST-P liver foci and preneoplastic liver lesions in rats. Through the reduction of cell proliferation and without alteration of the apoptosis process, ELF-EMF interferes with the altered cell cycle continuity and DNA synthesis induced by ChemIndHep. Theoretically, we found that with the use of a 120 Hz ELF-EMF it was possible to achieve modulation of the magnetic-sensitive short-lived RP intermediaries produced by the OS, generated when xenobiotics are metabolized during the catalytic cycle by the CYP450 in the early ChemIndHep. The process of modulation uses the competitive kinetics of the RP selective reactions [71], giving the role of a molecular motor to the enzymatic protein [9]. To achieve this, we studied a quantum mechanics model to describe the interaction of RP/hepatocytes following the typical Haberkorn approach studying the spin dynamics and employing the path integral method and second quantization. The idea of this chapter was to obtain an explanation of the way in which hepatocytes can modify their electronic structure when interacting with the RP, which comes from the enzymatic reaction. Although it is an in-depth mathematical model, the results of our research provide us with further details of the MF's control on BS, specifically in the ChemReac that modulates the electrons in OS (cytoprotective effect) generated in the reactive hepatocytes-RP system in the liver, with the outcome of understanding hepatocarcinogenesis.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Chapter 4

Vitamin E and Influenza Virus Infection

Milka Mileva and Angel S. Galabov

Additional information is available at the end of the chapter

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Abstract

Influenza is an infectious disease causing huge medical and economic losses. Influenza pathogenesis is associated with two processes in the human body: (i) lung damage due to viral replication in the columnar ciliary epithelium of bronchi and bronchioles and (ii) inflammatory burst inducing an increase in reactive oxygen species generation that causes extensive damage in cellular membranes of the small vessels. The oxidative stress in influenza virus-infected organism provokes free-radical oxidation of unsaturated lipid chains in the cell membranes. As vitamin E is a lipid-soluble substance and possesses a hydrophobic tail, it tends to accumulate within lipid membranes. There, it acts as the most important chain breaker, reacting with lipid peroxyl radicals much faster than they can react with adjacent fatty acid side chains. Among the antioxidants tested in influenza virus infections in mice, vitamin E occupies the leading position because of its efficacy in preventing oxidative damage through its free-radical scavenging activity. Although vitamin E is not possessing specific antiviral action, its antioxidant effect probably plays important role in lung and liver protection. Attention should be paid to the synergistic character of antiviral effect of the combination vitamin E and oseltamivir. Vitamin E could be recommended as a component in multitarget influenza therapy.

Keywords: flu pathogenesis, oxidative stress, vitamin E, combination with antivirals, influenza therapy

1. Influenza

Influenza is an acute infectious disease that exerts a very great effect on human society, causing huge medical and economic losses. Influenza usually occurs in annual seasonal (winter) outbreaks or epidemics (in moderate temperature climates). Moreover, influenza pandemics periodically attack the populations of all continents. People of all ages are affected, but the prevalence is greatest in school-age children. The disease's severe course, complications, and

mortality are greatest in infants, the elderly, and those with underlying illnesses—chronic pulmonary or cardiovascular diseases, and diabetes mellitus. The severe complications include hemorrhagic bronchitis or pneumonia (primary viral or secondary bacterial). In addition, fulminant fatal influenza viral pneumonia can occur, with death proceeding in as little as 48 hours after the initial flu symptoms [1]. The World Health Organization recommends influenza vaccines as a main tool for preventing infection and anti-influenza chemotherapeutics with antiviral drugs for treatment and/or prophylactically [1]. The antivirals effective against influenza are divided into two types based on their modes of action: (i) inhibitors of the neuraminidase—oseltamivir, zanamivir, peramivir, and related compounds, efficacious against influenza A and B virus infections, and (ii) blockers of the protein M2—rimantadine-HCl and amantadine-HCl, active against influenza virus A infections. Although both types of agents have proven antiviral effectivity, the rate of drug resistance is constantly increasing, especially for M2 blockers [2].

Two principal problems are related with vaccine prevention: (i) anti-influenza vaccines commonly demonstrate 70–90% effectivity in young persons, with rates markedly decreasing in the elderly; (ii) the protection length is limited to a few months or a season because of the continuous viral antigenic drift based on gradually accumulated mutations, requiring annual revaccination [3].

2. Influenza pathogenesis

The pathogenesis of influenza virus infection is associated with two general processes in the human body: (i) local lung damage due to viral replication in the columnar ciliary epithelium of bronchi and bronchioles, which leads to progressive damage of the alveolar cells, bronchopneumonia (viral or combined viral-bacterial), massive bronchitis (including bronchiolitis), and the like, as the major causes of death [4]; (ii) a dramatic inflammatory burst that induces among other processes an increase in reactive oxygen species generation, causing extensive damage in cellular membranes, predominantly in the small vessels, arterioles, and capillaries [5–8]. In addition, extrapulmonary complications affect many organs and tissues, such as heart, brain, middle ear, liver, and endocrines, and even stomach and kidneys, though that is rare [9–14].

2.1. Respiratory tract damages

Influenza virus replicates in the respiratory tracts of humans, mainly in the lungs. Extrapulmonary multiplication of this virus has not been proven in people with influenza, nor in experimental conditions in influenza virus-infected laboratory animals. Influenza virus replicates throughout the whole respiratory tree. Tracheobronchitis is the common clinical picture of influenza. In the acute stage, multifocal destruction and desquamation of the columnar epithelium of the trachea and bronchi accompanied with edema and congestion of the submucosa are characteristic. In about 50% of cases, tracheitis and bronchitis have a hemorrhagic character. Cell necrosis is the final stage of desquamation of the affected epithelium

with concomitant attainment of the mucus glands. Small- and medium-sized bronchioles are strongly affected by the processes seen in the larger airways, with an entirely necrotic bronchiolar wall associated with polymorphonuclear cell infiltrate. Influenza virus pneumonia very often proceeds to secondary bacterial pneumonias. Destruction of alveolar epithelium and endothelium can worsen the severity of lung injury [4, 15].

Lung disorders in influenza virus infection may be triggered by: (i) a massive infiltration of leukocytes, mainly polymorphonuclear leukocytes, into the alveolar space; (ii) a decrease in the partial pressure of oxygen, causing the development of hypoxia; (iii) an increase in the partial pressure of CO_2 and development of metabolic acidosis; (iv) a "cytokine storm"—a release of cytokines, eicosanoids and prostaglandin E2 and an enhanced immune response; and (v) the development of oxidative stress [5, 7, 16, 17].

Our previous data and that of most of the literature showed that experimental influenza virus infection in susceptible laboratory animals (mice and ferrets) imitates the above influenza clinical picture: progressive damage of the alveolar cells, acute inflammatory reaction, and development of massive bronchitis and probably pneumonia, in parallel with a decrease in endogenous lipid- and water-soluble antioxidants levels, as well as the compensatory changes of antioxidant enzyme activities [18–21].

2.2. Oxidative stress in influenza virus infection

Lungs are the target organs of the influenza virus. However, in the course of influenza virus infection, dynamic changes in oxidative metabolism, provoked by the overgeneration of ROS (reactive oxygen species) and the activation of neutrophils, can reach the development of oxidative stress [5, 18, 19, 22–24]. Oxidative stress is defined as a disturbance of the prooxidant-antioxidant balance in favor of prooxidants. Influenza viruses are known to induce ROS-generating enzymes and to disturb antioxidant defenses [5, 18, 19, 25], causing changes in antioxidant enzyme activity [5] and decreases in endogenous low-molecular-weight antioxidants. Overgeneration of ROS may influence signaling pathways by activating "redox switches" [26]. In lungs, redox homeostasis is crucial in the pathology of influenza because it is associated with cytokine production, inflammation, cell death, and other pathological processes that could be triggered by enhanced ROS generation (**Figure 1**).

Since the products of oxidative stress possess high cytotoxic activity, it is very important to study mechanisms of detoxification in the infected body. After the epithelial cells are infected, tissue-resident alveolar macrophages are the first responders to viral infection in the lungs (**Figure 1**). They can promote viral clearance through the phagocytosis of viral particles or infected apoptotic cells (efferocytosis) and the release of a plethora of inflammatory cytokines and chemokines to initiate and drive the immune response [27–30] . Due to the ability of ROS to react with almost any kind of biological molecule, including proteins, lipids, and nucleic acids, their elevation is generally associated with genome instability, dysfunction of organelles, and apoptosis [31].

Antioxidant defense mechanisms, including enzymes like superoxide dismutase, catalase, and small molecules such as vitamins C and E and glutathione, protect tissues against oxidants [32].

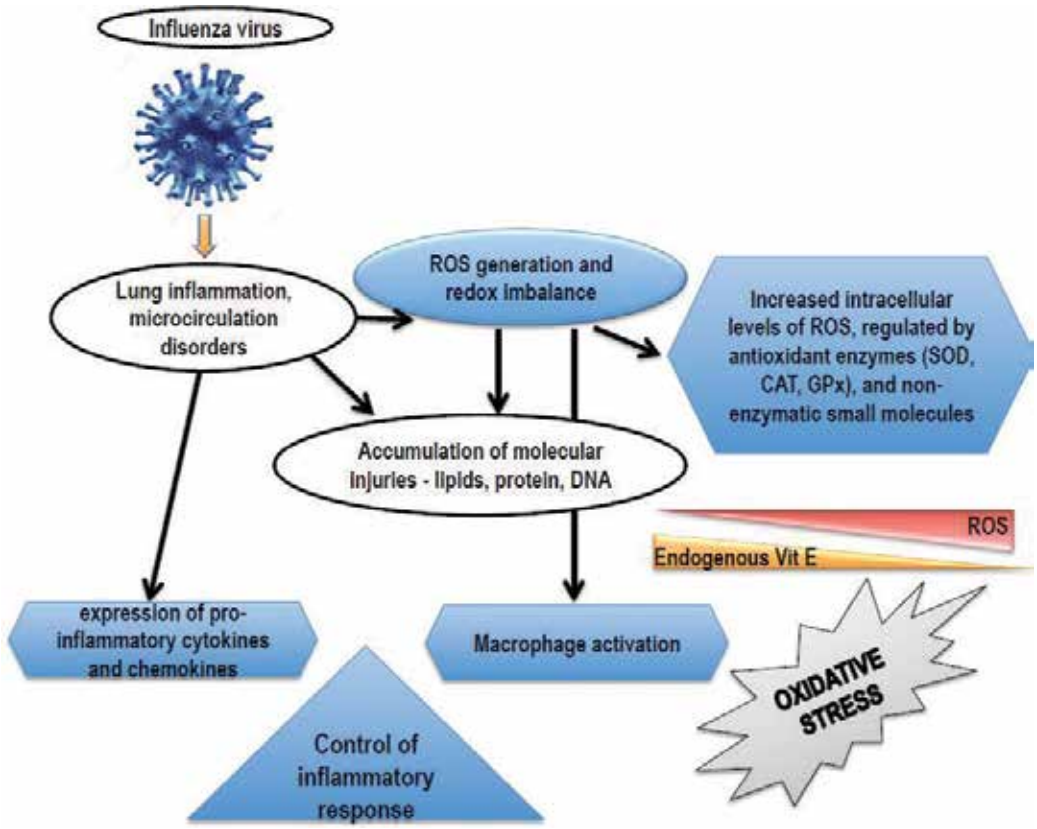

Figure 1. Respiratory tract damages, causes by influenza virus infection.

Vitamin E is the most active natural fat-soluble antioxidant capable of protecting unsaturated fatty acids in cellular membranes from peroxidation, thereby contributing to membrane stability [33]. Both human clinical trials and animal studies have shown a beneficial effect of supplemental vitamin E on the immune system [34].

Studies in the last decade established that the nuclear factor (erythroid-derived 2)-like 2 (NRF2) encoded in humans by the NSF2 gene, is a protein regulating the expression of antioxidant proteins that protect against oxidative damage triggered by injury and inflammation. NRF2 controls the basal and induced expression of antioxidant response element-dependent genes to regulate the physiological and pathophysiological outcomes of oxidant exposure. NRF2 has a substantial impact on oxidative stress and toxicity, regulating the antioxidant defense [35].

At this point of view, the oxidative stress is caused by the imbalance between production of reactive oxygen species (ROS) and the body's ability to detoxify the reactive intermediates.

Recent studies described the role of NRF2 gene coded protein in the development of oxidative stress. The antioxidant pathway controlled by NRF2 is pivotal for protection of lungs against the development of influenza virus infection-induced pulmonary inflammation and injury under oxidative conditions. The NRF2-mediated antioxidant system is essential to protect the lungs from oxidative injury and inflammation induced by influenza virus infection [36, 37].

3. Vitamin E and influenza virus infection

It has been proven that oxidative stress in the influenza virus-infected organism provokes free-radical oxidation of unsaturated lipid chains in the cell membranes (lipid peroxidation), which reduces their permeability as a whole. In the presence of antioxidant deficiency, as described below, when all cell membranes are exposed and/or damaged, influenza infection proceeds with severe pathology and results in serious damage at all levels in the body [38].

It was established that, during influenza infection in mice, the activity of antioxidant enzymes SOD and catalase were changed, along with a decrease of the amounts of endogenous low-molecular-weight antioxidants such as α-tocopherol (**Table 1**), glutathione, and ascorbate [19, 24, 39–41],). Endogenic levels of vitamin E were significantly decreased in lung, liver, and blood plasma [19, 23, 42]. In addition, changes in cytochromes were recorded as well as decreases in the activities of liver cytochrome P-450-dependent monooxygenases [18, 43]. Together, these facts indicate that, in the course of the disease, the buffering capacity of the organism's antioxidant protection diminished [18, 19, 22, 23, 25].

These data demonstrate that, during influenza virus infection, a decrease in natural antioxidant vitamin E was established, accompanied by a significant increase in endogenous lipid peroxidation products.

Oxidative damage in the course of influenza virus infection is quite large, even when registered in experimental animals (mice) at low virus-inoculation doses. In conditions involving non-infected animals with suppressed antioxidant defense systems, the consequent inoculation of influenza virus resulted in an acceleration of oxidative stress and graduated tissue damage.

Different conditions can favor the host's susceptibility to influenza virus infection; among them are cold exposure and stressors of physical, chemical, and psychological origin. For example, immobilization and cold-restraint stress are widely used experimental models that are accompanied by a considerable decrease in the antioxidative capacity of the animal organism; they are also used for the indirect modulation of antioxidant deficiency in experimental animals [19, 44–47].

Because of the significant role of oxidative stress in the pathogenesis of influenza virus infection, a lot of work has been done to test the influence of antioxidants on the course of influenza. Drugs stimulating NRF2 pathway are tested for treatment of diseases causing oxidative

Group	Lung		Liver		Blood plasma	
	5th day	7th day	5th day	7th day	5th day	7th day
I Control	2.2 ± 0.31	2.14 ± 0.26	4.94 ± 0.51	5.4 ± 0.42	1.8 ± 0.065	1.72 ± 0.07
II Flu	1.47 ± 0.14	1.7 ± 0.17	3.35 ± 0.42	3.12 ± 0.37	1.46 ± 0.035	1.2 ± 0.37

Values are expressed as means ± SEM [41].

Table 1. Endogenous content of vitamin E [nmol/mg protein] in lung, liver, and blood plasma of mice experimentally infected with influenza virus A/Aichi/2/68 H3N2 (1.5 MLD_{50}).

stress, influenza virus infection included [48]. Experiments on in vivo models, predominantly in mice, hold a significant place in such investigations.

Among the antioxidants tested against influenza virus infections in mice [17, 49–52] α-tocopherol (vitamin E) occupies the leading position. This is because of its efficacy in preventing oxidative damage through its free-radical scavenging activity [16, 18, 24, 42, 53–55].

Protein expression of NRF2 is found to be increased in both the cholesterol-fed and the vitamin E-supplemented rabbits via activation of NRF2 pathway, resulting in induction of several antioxidant genes. Vitamin E appeared to afford the protection effect of NRF2 [48, 55]. Besides, it was found that vitamin E prevents the NRF2 suppression by allergens in alveolar macrophages, proved for asthmatic model in vivo [56, 57].

These data clearly show the role of antioxidants, such as vitamin E, which can be manifested in several ways: (i) to capture free radicals in enzymatic or non-enzymatic mechanism(s), (ii) to suppress their generation, and (iii) to affect these processes in an indirect way, for example, by inhibiting viral replication.

As vitamin E is a lipid-soluble substance and possesses a hydrophobic tail, it tends to accumulate within the interior of lipid membranes. There, it acts as the most important chain-breaker, as it reacts with lipid peroxyl radicals about four times faster than they can react with adjacent fatty acid side chains. It is well known that vitamin E is able to prevent oxidative damage [58–61], because its lipophilic structure contributes to easy and passive diffusion through the cell membranes, allowing it to reach the mitochondria and the single-plated reticulum. In this way, vitamin E protects them against lipid peroxidation and damage (**Figure 2**). Especially important is its termination of free-radical chain reaction, which protects membrane polyunsaturated fatty acids from oxidation involving reactive oxygen species [61].

Vitamin E is known to affect inflammatory responses in different tissues, including the lung, not only via direct quenching of oxidative stress [42, 62], but also through modulation of oxidative eicosanoid pathways and prostaglandin synthesis [58, 63, 64], inhibition of inflammatory mediators [59], and control of apoptotic lipid signaling [60]. A stabilizing role of Vitamin E has a stabilizing role for membrane phospholipids [61] (**Figure 2**).

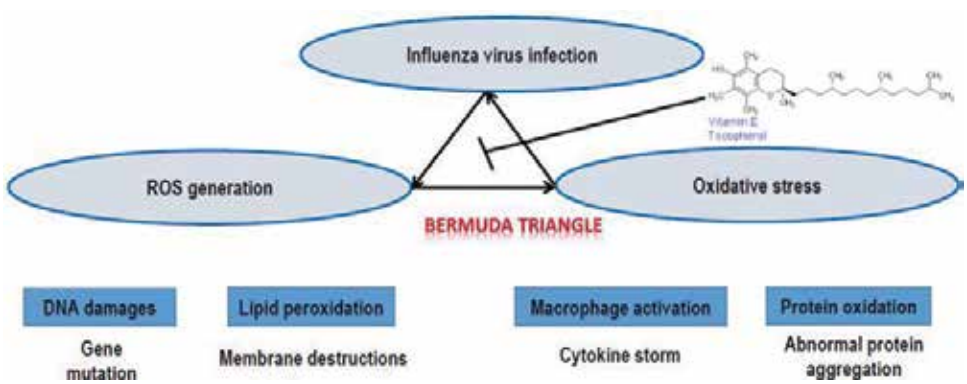

Figure 2. "Bermuda triangle" composed by the pathogenesis of influenza virus infection in the infected body. Vitamin E action is directed to the storm center.

Several different non-antioxidant functions of vitamin E may be essential for the maintenance of cell integrity and functions, such as its role as an anti-phospholipase A2 agent, that is, as a stabilizer of the lipid bilayer of membranes against hydrolyzed and oxidized lipids [65].

The *in vivo* investigations on influenza virus-infected laboratory animals and the clinical data on influenza patients revealed a negative correlation between pulmonary inflammations and endogenous levels of vitamin E in the body. Exogenous vitamin E supplementation has been tied to reducing severe symptoms of lung disease [16, 18, 24, 66].

It is clear that influenza virus infection is a powerful prooxidant that causes a significant increase in lipid peroxidation products in lung, liver, and blood plasma as well as a decrease in natural antioxidants (vitamin E, glutathione) and cytochrome P-450 (CYP). Moreover, in the liver, cytochrome *c* reductase and liver monooxygenases (aniline hydroxylase, ethylmorphin-N-demethylase, analgin-N-demethylase, and amidopyrine-N-demethylase) are inhibited as compared to their activity in control (non-infected) animals.

4. Effects of vitamin E supplementation

As mentioned above, investigations on mice experimentally infected with influenza virus found that endogenous vitamin E content was significantly decreased after influenza virus inoculation. In addition, the amount of cytochrome P-450 in the liver and the activity of cytochrome *c* reductase decreased by about two times on the 5th–7th days post virus inoculation. The decrease in cytochrome P-450 was found to correlate with increases in the concentration of lipid peroxidation products in liver, lung, and blood [18]. Influenza virus infection significantly inhibits liver monooxygenase activity. As a consequence, products from the decreased enzymatic function accumulate in the liver, resulting in the destruction of cytochrome P-450 and its transformation to the catalytically inactive P-420 form.

The effects of influenza virus infection on liver monooxygenases and lipid peroxidation are different from the effects of the hydrophobic xenobiotic substrates of cytochrome P-450. Evidently, the oxidative stress induced in the liver by hydrophobic xenobiotics is a consequence of enhanced oxidation by cytochrome P-450-dependent monooxygenases. The decrease in liver monooxygenase activity resulting from influenza virus infection is accompanied by increases in lipid peroxidation products in the liver, which is not a result of activation of cytochrome P-450-dependent monooxygenases. It may be presumed that influenza virus induces free-radical processes outside the liver, thus producing free radicals and/or activated oxygen species. These reactive compounds must diffuse or be transported over the hepatocyte barrier to initiate lipid peroxidation in the liver.

The protective effect of vitamin E against lipid peroxidation was dose-dependent and was more pronounced on the 5th day as compared to the 7th day after virus inoculation [18, 22]. This agrees with data from Peterhans [5] and Jacoby and Choi [38]. Vitamin E supplementation led to stabilization of cytochrome P-450. Concentrations of the hepatic cytochrome P-450 in infected mice reached the values found in control (non-infected animals) after vitamin E supplementation (120 or 240 mg/kg b.w.), because the monooxygenase activities were restored.

However, researchers have shown that endogenic levels of vitamin E are significantly decreased in lung, liver, and blood plasma (**Table 1**) during the course of flu infection [21, 23, 24, 42]. Animal and human studies have demonstrated a negative correlation between endogenous levels of vitamin E in the body and pulmonary inflammations, and exogenous vitamin E supplementation has been tied to reducing severe symptoms of lung disease [16, 18, 64].

It is well known that vitamin E is able to prevent oxidative damages [58–60].

5. Vitamin E in the influenza therapy

Currently, treatment of influenza is directed mainly at targeting the first pathogenetic component through administration of specific antivirals. Application of correctors of influenza pathogenesis that are associated with controlling inflammation and oxidative stress remains in the background.

Among the antioxidants tested in influenza virus infections in mice [17, 49–51, 65], α-tocopherol (vitamin E) occupies the leading position because of its efficacy in preventing oxidative damage through its free-radical scavenging activity [16, 18, 24, 42, 53–55, 66, 67].

Although vitamin E is not an agent with specific antiviral action, its antioxidant effect probably plays an important role in liver protection.

The most important question is whether vitamin E, as a natural antioxidant, could be used as anti-influenza agent.

In fact, the ideal protective agent against flu should fulfill several criteria: (a) it must not allow the formation of resistant viral strains; (b) it must have a general protective effect on the majority of organs; (c) it must have an acceptable toxicity profile and protective time-window effect; and especially importantly, (d) it must provide strong protection against the symptoms of emerging influenza, such as the oxidative state of the infected body.

Evidently, a more effective treatment strategy is needed. Immunomodulators have been proven to be highly successful in treating the flu, at least in mouse infection models [68–71]. Using antioxidative agents to act directly on downstream deleterious inflammation events is also of significant importance in flu therapy.

The preventive effects of vitamin E and vitamin C, alone and in combination, was tested on the damage caused by influenza virus infection [72]. Mice, infected with influenza virus A/2/68/(H3N2) (1.5 LD_{50}), were administered once-daily doses of vitamin E (60 mg/kg b.w.) and vitamin C (80 mg/kg b.w.) intraperitoneally (for 3 days before virus inoculation). Vitamin E effectively restored lipid peroxidation levels increased by influenza virus infection. The effect of vitamin C was similar, but slighter. The combination (vitamin E + C) had a greater effect on lipid peroxidation levels than did their separate administration. P-450-dependent monooxygenase activity was significantly restored, and more pronounced cytochrome P-450 content and NADPH-dependent cytochrome *c* reductase activity was noted. The preventive effect of vitamin E was stronger than that of vitamin C, but the combination (vitamin E + C)

had the strongest effect. The superior protective effect of the combination is probably due to the better interaction between hydrophobic and hydrophilic low-molecular-weight antioxidants against a free-radical disease like influenza. The mechanism of this interaction is related to vitamin C's ability (when situated in aqueous phase) to recycle vitamin E (located in membranes), repairing vitamin E's tocopheroxyl radical. Thus, vitamin C promotes the function of vitamin E as a free-radical scavenger [73, 74].

An underappreciated approach in flu therapy continues to be combination administration regimens of specific viral replication inhibitors together with antioxidants. Therefore, investigations on the combination effects of specific anti-influenza chemotherapeutic agents and antioxidants are of special interest. Previously, we established a favorable combination effect of the antioxidant 4-methyl-2,6-ditretbutylphenol (ionol) with M2-blocker rimantadine in mice infected with influenza virus A(H3N2). Ionol was administered intraperitoneally in a 3-day course (45 or 75 mg/kg daily) before virus inoculation, and rimantadine (oral application of 15 mg/kg) was administered for 5 days following the day of infection [75].

Recently, a strong beneficial effect of the combination of α-tocopherol (a component of vitamin E) and oseltamivir was demonstrated in the treatment of experimental infection with influenza virus A/H3N2 in mice [76]. The results showed that this combination of agents simultaneously suppressed the two main processes in the pathogenesis of influenza—the development of pulmonary lesions in the respiratory tract as a result of virus replication and the oxidative stress damage to membranes of small vessels and other tissues in the body—thus characterizing it as a very good prospect for flu therapy.

However, a question arose: Is oseltamivir an antioxidant? We used some model systems to test oseltamivir's ability to scavenge superoxide radicals, to inhibit their generation, and to influence Fe^{2+} or (Fe^{2+}-EDTA)-induced lipid peroxidation in liposomal egg suspension and in lung and liver microsomes [77]. We concluded that the reduction of oxidative stress in vivo is not connected with oseltamivir's effect on the development of free-radical processes in the organism. Oseltamivir's effect on oxidative stress in the course of viral infection could be explained by its specific therapeutic effect, which is connected with suppression of viral replication in the target organ.

The in vivo antiviral activity of the combination vitamin E + oseltamivir, expressed by a marked protective effect on the survival of influenza A virus-infected animals, was recorded when vitamin E was administered simultaneously with oseltamivir phosphate via a 5-day course post virus inoculation [76]. This effect was not observed when the vitamin E course started 120 or 48 hours before viral inoculation. According to this study, vitamin E applied individually had no effect on the course of influenza A virus infection caused by 10 MLD_{50}. Only a lower value of the lung index was registered. In our previous study, we established a protective effect of vitamin E at virus infection with 2 MLD_{50} [18, 24].

Special attention should be paid to the sharp synergistic character of the antiviral effect of the combination vitamin E and oseltamivir at a dose of 0.625 mg/kg administered simultaneously, which resulted in the following: (i) a pronounced increase in the protection index, attaining 76%, and a lengthening of the MSD by 3.2 and 4 days; (ii) a pronounced decrease in

lung infectious virus titer; and (iii) a strong reduction in lung lesions. Oseltamivir at the same dose applied separately did not manifest antiviral activity.

The observed phenomenon of a strong oseltamivir dose-dependence of the combined anti-flu effect attaining a pronounced synergism at the lowest tested dose of 0.625 mg/kg merits a special attention. One explanation for this phenomenon could be the interaction of these two agents related to their specific mechanisms of action on the viral target structures in the lung. It is well known that vitamin E is included in the cellular lipid bilayer, thus decreasing cellular membrane permeability [60, 62, 64]. Oseltamivir, for its part, mimics cellular neuraminic acid, thus interfering with the exit process of the new progeny virions [78, 79]. These two processes run in parallel, thus opposing the virus infection course on the cellular level. The two substances most likely compete in the modification of cellular membranes through their specific mechanisms of action. Therefore, the place of emerging synergism for oseltamivir and vitamin E is most likely the cell membrane in the viral target area in the lung.

The favorable (even synergistic) type of interaction between vitamin E and oseltamivir was absent when vitamin E was administered for 5 days before virus inoculation—that is, before the onset of the oseltamivir course.

The described results suggest that vitamin E has an important place as a component of the complex therapy of epidemic flu when administered simultaneously with chemotherapeutic agents, such as neuraminidase inhibitors. Moreover, in addition to its membrane protective effect in the influenza virus target area, vitamin E manifests pronounced activities as an antioxidant agent and as a protein kinase C inhibitor and a protector of lung tissue during inflammatory lung illnesses [59, 80]. The study discussed above [76] convincingly demonstrates a strong beneficial effect of the combination of vitamin E and oseltamivir in the treatment of experimental infection with influenza virus A/H3N2 in mice.

The results show that this combination of agents simultaneously suppresses the two main processes in the pathogenesis of influenza, the development of pulmonary lesions in the respiratory tract resulting from virus replication and the oxidative stress damage to the membranes of small vessels and other tissues in the body, thus characterizing it as a very likely prospect in the therapy of flu.

In summary, vitamin E could be recommended as a reliable agent, a component in multitarget influenza therapy.

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Chapter 5

γ-Tocotrienol Reversal of Epithelial-to-Mesenchymal Transition in Human Breast Cancer Cells is Mediated through a Suppression of Canonical Wnt and Hedgehog Signaling

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Additional information is available at the end of the chapter

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Abstract

γ-Tocotrienol, a natural isoform within the vitamin E family of compounds, displays potent antiproliferative, apoptotic and reversal of epithelial-to-mesenchymal transition (EMT) activity against breast cancer using treatment doses that have little or no effect on normal cell viability. EMT is a route by which epithelial cells undergo various biochemical alterations leading to the acquisition of mesenchymal traits. Several aberrant signaling pathways are involved in EMT-dependent cancer metastasis. Specifically, dysregulation of the canonical Wnt and Hedgehog pathways are intimately involved in promoting breast cancer EMT and metastasis. Therefore, studies were conducted to examine effects of γ-tocotrienol on Wnt and Hedgehog signaling. Results from these studies demonstrate that γ-tocotrienol significantly inhibits canonical Wnt and Hedgehog signaling by inhibiting receptors, co-receptors and ligand expression, as well as inhibiting expression of cytosolic and nuclear signaling proteins within these pathways. Additional studies showed that γ-tocotrienol treatment increased the expression of negative regulators of both the Wnt and Hedgehog pathways. These findings demonstrate that γ-tocotrienol reversal of EMT is mediated, at least in part, through the inhibition of canonical Wnt and Hedgehog signaling, and strongly suggest that this form of vitamin E may provide significant benefit in the prevention and treatment of metastatic breast cancer.

Keywords: γ-tocotrienol, epithelial-to-mesenchymal transition, canonical Wnt pathway, canonical hedgehog pathway, breast cancer

1. Introduction

Breast cancer is the second leading cause of death in women, and it originates from malignant breast cancer cells displaying unregulated growth to produce a tumor mass [1, 2]. Several cellular mechanisms are dysregulated in breast tumor cells, including the canonical Wnt and Hedgehog signaling pathways, which play an important role in promoting oncogenic proliferation, survival, motility, invasion, and epithelial-to-mesenchymal transition (EMT) [3]. Although these events are complex and poorly understood, recent findings show that specialized cell membrane microdomains known as lipid rafts are involved in mediating membrane receptor activation and signal transduction. Lipid rafts are solid platforms in the plasma membrane that consist of cholesterol and sphingolipids. Lipid rafts are essential for cellular signaling by recruiting transmembrane receptors with adaptor and signaling proteins from non-rafts to the raft area of the cell membrane [4–6]. In the case of canonical Wnt and Hedgehog signaling, low-density lipoprotein receptor-related protein 6 (LRP6) and patched (PTCH2), the main receptors for activation of these signaling pathways, were shown to be primarily located in the lipid raft microdomain [7–9]. Lipid rafts have been shown to be essential for Hedgehog signal transduction [10]. γ-Tocotrienol is a natural vitamin E isoform that displays potent anticancer activities [11–13]. Previous reports have clearly shown that γ-tocotrienol exerts antiproliferative and apoptotic activity against neoplastic mammary epithelial cells at treatment doses that had little or no effect on normal cell growth and viability [14, 15]. The anticancer effects of γ-tocotrienol appear to be mediated through a variety of intracellular signaling mechanism [16–18]. Recently, γ-tocotrienol was found to disrupt lipid raft integrity and attenuation of receptor signaling transduction [19]. This chapter will focus of experimental evidence demonstrating γ-tocotrienol reversal of EMT is mediated through the inhibition of the canonical Wnt and Hedgehog signaling pathways.

2. Vitamin E and breast cancer

Epidemiological studies have shown that diet and nutrition can play a major role in cancer development and progression. It has been suggested that approximately 30–35% of cancer morbidity and mortality might be prevented with suitable adjustment of nutrition, and up to one third of all the cancers in the United States can be avoided by increasing the consumption of fruits and vegetables in the daily diet [20]. Vitamin E is a generic term that includes a family of eight naturally occurring compounds that are further divided into two subgroups known as tocotrienols and tocopherols. Tocotrienols are relatively rare and found only a few natural sources, such as palm oil, rice bran oil, and annatto bean, while tocopherols are much more abundant and found in a wide variety of foods, such as nuts, whole grains, dark green vegetables, egg yolk, and various vegetable oils [21–24]. The relative levels of tocopherol and tocotrienol in various dietary oil and fats are shown in **Table 1**.

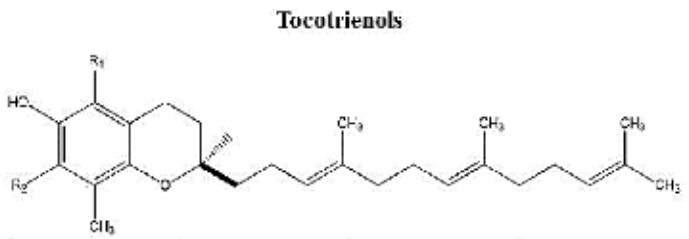
The chemical structure of all vitamin E isoforms are very similar and characterized by a long phytyl chain linked to a chroman ring structure methylated to varying degrees at the 5, 7, and 8 positions. The four isoforms in each subclass are classified as α-, β-, γ-, and δ-tocotrienol

	Tocopherol	Tocotrienols			
Dietary oil	**α**	**α**	**γ**	**δ**	**Total tocotrienol**
Palm	152	205	439	94	738
Rice brain	324	236	349	–	586
Wheat germ	133	26	–	–	26
Coconut	5	5	1	19	25
Palm-kernel	12	21	–	–	21
Coco butter	11	2			2
Corn	112	–	–	–	0
Cottonseed	389	–	–	–	0
Peanut	130	–	–	–	0
Olive	51	–	–	–	0
Safflower	387	–	–	–	0
Soybean	101	–	–	–	0
Sunflower	487	–	–	–	0

Table 1. Vitamin E levels (mg/L) in common dietary oils [21].

or tocopherol. Tocotrienols differ from tocopherols only in that they contain an unsaturated, whereas tocopherols contain a saturated phytyl tail. **Figure 1** shows the chemical structures of different isoforms of tocotrienols.

Interestingly, numerous studies have shown that tocotrienols, but not tocopherols, have selective antiproliferative and apoptotic effects against various forms of breast cancer, while have little effect on normal mammary epithelial cell growth or function [14, 15, 25]. The anticancer



Isoforms	R_1	R_2	Phytyl chain
α-Tocotrienol	CH_3	CH_3	Unsaturated
β-Tocotrienol	CH_3	H	Unsaturated
γ-Tocotrienol	H	CH_3	Unsaturated
δ-Tocotrienol	H	H	Unsaturated

Figure 1. General chemical structure of the different tocotrienols isoforms.

potency of different tocotrienol isoforms was determined to be characterized as δ-tocotrienol ≥ γ-tocotrienol > α-tocotrienol > β-tocotrienol [11, 15, 26]. The anticancer effects of tocotrienols were discovered in nutritional studies that investigated the role of high-dietary fat consumption on the development of mammary tumorigenesis in laboratory animals. These studies showed that diets containing high levels of palm oil inhibited the carcinogen-induced mammary cancer in rats [27]. Additional studies showed that palm oil diets stripped of tocotrienol no longer displays their protective effect against mammary tumorigenesis.

During the past decade, tocotrienols have received a great deal of attention because of their potential value in the prevention and treatment of breast cancer. Tocotrienols have been shown to inhibit multiple intracellular signaling pathways in cancer cells [15, 28]. Specifically, tocotrienols have been found to suppress EGF-dependent mitogenic signaling in neoplastic and normal mammary epithelial cells by significantly inhibiting activity of the phosphatidylinositol-4, 5-bisphosphate-3-kinase/protein kinase B (PI3K/Akt) pathway [29]. Other studies have shown that γ-tocotrienol treatment induced a dose and time-dependent inhibition of EGF-dependent Akt phosphorylation (activation) in mammary tumor cells, and these effects were not found to be associated with an increase in tensin homolog (PTEN) or protein phosphatase 2 A (PP2A) activity [30]. γ-Tocotrienol was also found to decrease activity of signaling proteins downstream of Akt, such as inhibiting the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cell (NFκB) by suppressing the activation of inhibitor of nuclear factor kappa kinase alpha and beta (IKKα and IKKβ), enzymes associated with induction of the NFκB activation [30]. Inhibition of NFκB transcription is associated with a suppression in cell proliferation and survival [31]. Additional studies have shown that the antiproliferative effects of tocotrienols is associated with an inhibition of protein kinase C alpha (PKCα) activation in breast cancer cells [32]. In addition, mitogen activated protein kinase (MAPK) has also been shown to be a target of γ-tocotrienol anticancer activity. Studies have indicated that γ-tocotrienol induced inhibition of EGF-dependent proliferation of preneoplastic CL-S1 mouse mammary epithelial cells resulted from an inhibition of G-protein-mediated activation of adenylyl cyclase, cyclic adenosine monophosphate (cAMP) production, as well as a reduction in phosphorylated (activated) extracellular signal-regulated kinase 1/2 (ERK1 and ERK2) [33]. In addition to the inhibition of mitogenic signaling, γ-tocotrienol is known to inhibit numerous vital cellular functions including inhibition of cell cycle progression [13], mevalonate pathway [34, 35], glycolysis [12], angiogenesis [36], and epithelial mesenchymal transition (EMT) [37].

Lipid rafts are distinct structures within the cell membrane that are enriched with sphingolipids, cholesterol, and acyl fatty acid chains that act to form a very rigid microdomain. Lipid rafts exist in two different forms: "planar lipid rafts," which are referred to as "non-caveolar" and caveolae lipid rafts. Planar rafts are characterized as non-invaginated microdomains lacking specific morphological features. In contrast, caveolae lipid rafts are tube-like invaginations of the plasma membrane characterized by specific scaffolding proteins or caveolins [4]. Some proteins are essential to membrane raft development and their role can be seen as constitutive components of rafts. One of the important proteins serving scaffolding functions in the caveolar raft is caveolin 1 (Cav1), a classical hairpin protein that plays a role in caveolae-mediated signaling, endocytosis, and transport [4]. Recent studies have shown that tocotrienols act to disrupt lipid raft integrity and disrupt plasma receptor membrane receptor activation and signal transduction. These findings provide evidence to explanation the wide range of inhibitory

effects γ-tocotrienol has on numerous signaling pathways [19]. Molecular targets associated with tocotrienol anticancer activity are shown in **Table 2**.

Figure 2 shows the effects of γ-tocotrienol treatment on the growth of malignant and normal human breast cancer cells. Results show that exposure various doses of γ-tocotrienol induced a dose-dependent inhibition in the growth of the highly malignant MDA-MB-231 breast cancer cells, as compared to cells in the vehicle-treated control group in **Figure 2A**. The IC_{50} dose γ-tocotrienol in these studies was found to be approximately 5 μM. However, treatment with similar or even higher doses of γ-tocotrienol on immortalized normal MCF-10A mammary epithelial cell line was found to have little or no effect on cell growth or viability (**Figure 2B**) [14].

Molecular target	References
PI3K/Akt	[29]
PKCα	[32]
MAPK	[33]
Cell cycle	[13]
Mevalonate pathway	[34, 35]
Glycolysis	[12]
Angiogenesis	[36]
EMT	[37]
Lipid rafts	[19]

Table 2. Summary of some of the molecular targets associated with mediating the anticancer effects of tocotrienols.

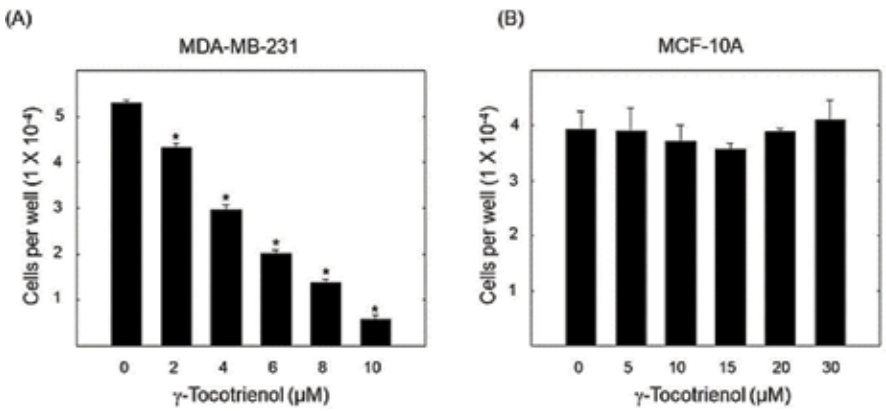


Figure 2. γ-Tocotrienol effects on the growth of the highly malignant MDA-MB-231 human breast cancer cells and the immortalized normal MCF-10A human mammary epithelial cells. MDA-MB-231 and MCF-10A cells were initially seeded at a density of 1×10^4 cells/well (6 wells/group) in 96-well culture plates and maintained on serum-free defined media containing 0–30 μM doses of γ-tocotrienol over a 4-day culture period. The viable cell number was determined by using the MTT colorimetric assay. Vertical bars show mean cell number ± SEM in each treatment group. (*$P < 0.05$) as compared with cells in their respective vehicle-treated control groups.

3. Epithelial-to-mesenchymal transition (EMT)

EMT plays a major role in organogenesis, angiogenesis and cancer metastasis [38, 39]. EMT was first observed and defined in the late 1980s at Harvard University by Elizabeth Hey [40]. EMT was defined as a differentiation program by which epithelial cells lose their attachment with other epithelial cells to become more mesenchymal-like, and are able to become mobile and invade their surrounding extracellular matrix [41, 42]. Because this process is reversible [43], epithelial cells displaying a mesenchymal phenotype, are also able to re-differentiate back into their epithelial phenotype [44]. Epithelial cells displaying their normal epithelial phenotype are well-structured in single layers of cuboidal or columnar cells. They are closely attached to surrounding cells by intercellular adhesion complexes. These cells also display an apicobasal polarity with a characteristic basal basement membrane that separates the epithelium from other tissues. In contrast, epithelial cells with a mesenchymal phenotype are characterized by the absence of polarity and intercellular adhesion junctions, hallmarks that have come to define EMT [45]. During the EMT process, cells lose the attachment of β-catenin and E-cadherin, which act to tightly link and attach surrounding epithelial cells together. This loss of attachment leads to a disruption of the adherens junctions [46]. These events then allow the mesenchymal phenotype to move freely and invade the surrounding extracellular matrix. In normal conditions, EMT provides a necessary function during embryogenesis, growth and wound healing. However, aberrant EMT can result in pathological conditions such as organ fibrosis and cancer metastasis. EMT mediated metastasis of malignant breast cancer epithelial cells can often form secondary tumors in the bone or lung [46]. EMT that occurs under normal conditions, such as embryogenesis, is referred to as type 1 EMT or classical EMT [47]. However, EMT that develops during inflammation, wound healing, tissue regeneration, and organ fibrosis is referred to as type 2 EMT, whereas EMT associated with cancer metastasis is termed type 3 EMT and plays an important role in the development, growth and progression of breast cancer [47].

Transcription factors also play a role in the initiation of EMT. Receptor activation by various growth factors, such as hepatocyte growth factor (HGF), epidermal growth factor (EGF), and fibroblast growth factor (FGF) are involved in the activation of various transcription factors involved in EMT. Growth factor-induced activation of transcription factors include zinc finger protein snail 1 (SNAIL1), (SNAIL2), zinc finger e-box-binding homeobox 1 (ZEB1), (ZEB2), twist, forkhead box protein 1 (FOXC1), (FOXC2), transcription factor 3 (TCF3), also known as (E47), and homeobox protein goosecoid (GSC) [37, 43, 45]. EMT also plays a role in the restructuring of extracellular matrix proteins by up-regulating fibronectin, collagen, proteases like MMPs, and other remodeling enzymes. In addition, autocrine and paracrine secretion of growth factors, cytokines, and extracellular proteins can modulate cancer cells phenotype and promote EMT [37, 43, 45].

Epigenetic modification, such as acetylation or methylation of the DNA, also can play a role in the EMT activation. For example, methylation of arginine (R531) by protein arginine methyltransferases 7 (PRMT7) plays a crucial role in inducing the EMT and the promotion of migratory and invasive behavior of breast cancer cells [48]. EMT can also be activated by expression of certain miRNAs, such as micro-RNA200/205 family (miRNA200) and (miRNA205), whose

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prominent targets are the ZEB1 and ZEB2, known as specific repressors of E-cadherin. Likewise, members of the ABC family of transporters, such as ABCB5, plays a major role in the activation of EMT [49]. Studies have shown that several signaling pathways, including the canonical Wnt pathway, the canonical Hedgehog pathway, Notch pathway, Janus kinase (JAK)/STAT pathway, and TGFβ pathway are involved in the activation of EMT [50]. Activation of these EMT-inducing signaling pathways leads to the disruption of adherens junctions (desmosomes), tight junctions, and gap junctions through suppression of several proteins, such as partitioning defective 6 homolog alpha or ZEB1, which represses plakophilin, an important junctional adhesion protein [43]. These pathways can act separately or together through cross-talk to increase cancer cell migration, invasion, drug resistance, stemness, and self-renewal potential [51, 52]. Taken together, it is clearly evident that EMT is an extremely complex process and a great deal more information is required to fully understand this phenomenon.

Figure 3 shows the effects of γ-tocotrienol on the expression of EMT cellular biomarkers in the highly malignant MDA-MB-231 human breast cancer cells. Western blot analysis shows that MDA-MB-231 cells in the vehicle-treated control group displayed relatively low levels of expression for the epithelial cell markers cytokeratin 8, cytokeratin 18 and E-cadherin, and corresponding high levels of expression for the mesenchymal cell markers vimentin, fibronectin and total β-catenin (**Figure 3A**). Treatment with 3–7 μM γ-tocotrienol (MDA-MB-231) induced a dose-responsive reversal in epithelial versus mesenchymal cell marker expression (**Figure 3A**). Immunocytochemistry was then performed to confirm the finding in **Figure 3A**.

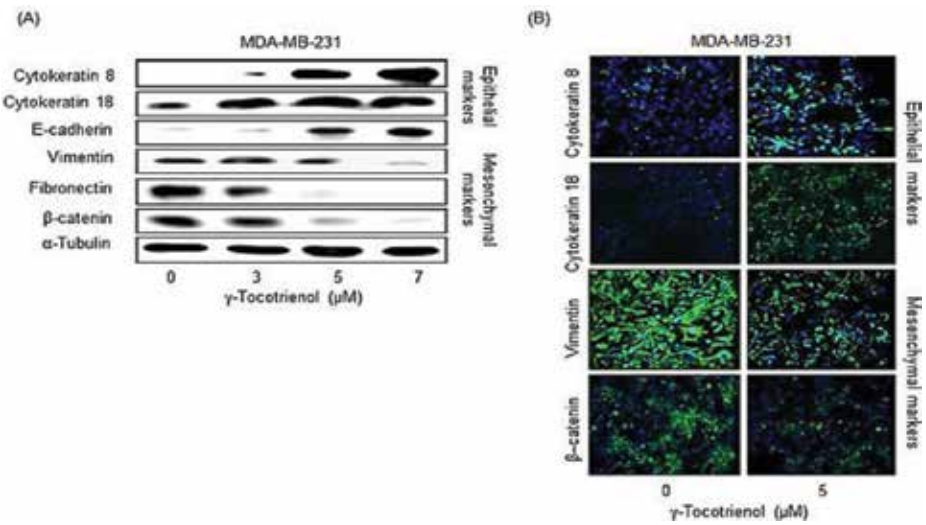

Figure 3. γ-Tocotrienol effects on epithelial versus mesenchymal cell markers expression. (A) Whole cell lysates were prepared from cells in each treatment group for subsequent separation by polyacrylamide gel electrophoresis (35 μg/lane) followed by western blot analysis. (B) Immunocytochemical analysis was done to confirm the finding shown in A. Cells in the various treatment groups were fixed, blocked, and incubated with specific primary antibodies for cytokeratin 8, cytokeratin 18, vimentin, and total level of β-catenin followed by incubation with Alexa Fluor 488-conjugated secondary antibody. Green staining in the photomicrographs (magnification 200×) indicates positive fluorescence staining for target proteins and the blue color represents counter staining of the cell nuclei with DAPI.

MDA-MB-231 cells in the vehicle-treated control group displayed a relatively low level of positive immunofluorescence staining for the epithelial cell marker cytokeratin 8, cytokeratin 18, and a relatively high level of positive immunofluorescence staining for the mesenchymal markers vimentin and β-catenin (**Figure 3B**). Treatment with 5 μM γ-tocotrienol resulted in a reversal of positive immunofluorescence staining of epithelial versus mesenchymal cell markers in MDA-MB-231 cells (**Figure 3B**) [14].

4. Canonical Wnt pathway

The canonical Wnt pathway is one of the fundamental pathways that is overexpressed in cancer metastasis that is involved in the initiation of EMT [53, 54]. Wnt is an acronym derived from two proto-oncogene wingless and intel 1. At present, 19 Wnt ligands have currently been identified [55]. Those ligands form a large group of secreted glycoprotein that are secreted through autocrine and/or paracrine mechanisms. After DNA transcription and translation takes place, protein is translocated to the endoplasmic reticulum (ER), where a lipid tail is attached to the ligand by porcupine enzyme. Then, the ligand is transported to Golgi apparatus by Wntless/evenness enzymes. At the Golgi, a sugar moiety is linked to the ligand, facilitating its translocation to the ECM and binding to the receptors, respectively. Once the Wnt ligand is in the ECM, numerous proteins, such as dickkopf (DKK1), sclerostin (SOST), secreted frizzled-related protein (SFRP), and Wnt inhibitory factor 1 (WIF1), play a role to block the signal. In contrast, other proteins, such as R-spondin (RSPO) and norrin can stimulate Wnt signaling [56]. Wnt pathway co-receptors are located in the lipid rafts which are microdomains in the cell membrane needed for the stimulation of signal transduction [7]. A number of proteins, such as glycosaminoglycan, dally and dly, are responsible for handling the Wnt ligand to the lipid rafts [55]. Ligand then bind to the co-receptors and induce activation of the signaling pathway [55].

The Wnt pathway can be stimulated canonically and non-canonically [57]. Nevertheless, the critical and most studied pathway is the canonical Wnt pathway, known to have a role in triggering EMT [58]. When there is no need for any developmental process, this pathway remains inactive and the receptor ligand is sequestered in the extracellular matrix by the action of number of specific binding proteins. Bound ligand to the Wnt receptor is inactive and prevents to a reduction in the phosphorylation of the disheveled protein (DVL), which is known to inhibit the cytosolic complex. The cytosolic complex composed of several proteins, such as glycogen synthase kinase 3 beta (GSK3β), axin 1, adenomatous polyposis coli (APC), and casein kinase 1 alpha (CK1α). The kinases in this complex remain active to phosphorylate the majority of the β-catenin, a biomarker for the canonical Wnt pathway activation. Phosphorylated β-catenin is then targeted for degradation by proteasomal enzymes [59]. In the nucleus, β-catenin is translocated out of the nucleus by the action of APC, Ran, and Manchette-associated binding adaptor protein 3 (BP3). The T-cell factor/lymphoid enhancer factor (LEF/TCF) area in the DNA is the binding location of β-catenin and is hidden by Groucho, histone deacetylase (HDAC), and glucose transporter-binding protein (GtBP) as a mechanism to rid the cell of β-catenin activity. Finally, the rest of β-catenin in the nucleus is sequestered by Chibby (CBY) and inhibitor of β-catenin and TCF 4 (ICAT) [60]. The summation of these events results in the blockade of Wnt signaling and downstream gene expression and mitogenesis.

However, during conditions of Wnt activation, such as during wound healing, the pathway becomes acutely active during the healing process. During this time, Wnt ligands are translocated to the extracellular matrix where they bind to their receptor and co-receptors, which ultimately leads to phosphorylation of DVL. Phosphorylated DVL block GSK3β activity in the cytosolic complex. As a result, β-catenin will not be phosphorylated and no longer targeted for degradation. The stabilized β-catenin can now be translocated from the cytosol into the nucleus and induce transcription [61]. There are also numerous other proteins such as CREB-binding protein (CBP), polymerase associated factor 1 (PAF1), and Brahma (Brm), which work together as transcription factors to potentiate the Wnt signaling pathway [60]. Activation of this pathway leads to increase cyclin D1 expression, which is associated with cell cycle progression and growth. Similarly, an increase in myelocytomatosis (c-Myc) expression as a result of Wnt activation leads to increased cell proliferation and increase in MMP9 expression, which is involved in the disruption of the tight junctions [62]. An increase in snail and slug expression leads to a loss of the attachment of β-catenin and E-cadherin and the progression of EMT [63]. However, after the wound is healed and Wnt signaling is no longer needed, a negative feedback effect can occur by the action of certain proteins, such as DKK1 and axin 2, and represents highly controlled gene expression and cell growth [64]. However, cancer cells are characterized by an increased expression of Wnt ligands, as well as has numerous proteins in the cytosolic complex, such as β-catenin, APC, or axin 1, that can become mutated. These factors lead to the continuous activation of the Wnt pathway and is associated with increased tumor growth, motility, invasion and metastasis [7, 65].

Figure 4 shows the effects of γ-tocotrienol treatment on the relative levels of signaling and regulatory proteins within the canonical Wnt and Hedgehog pathways. Total levels of the Wnt3a, FZD7 receptor, phosphorylated-LRP6 (active form), DVL2 and cyclin D1 were highly expressed in the vehicle-treated MDA-MB-231 cell line with corresponding relatively low expression of

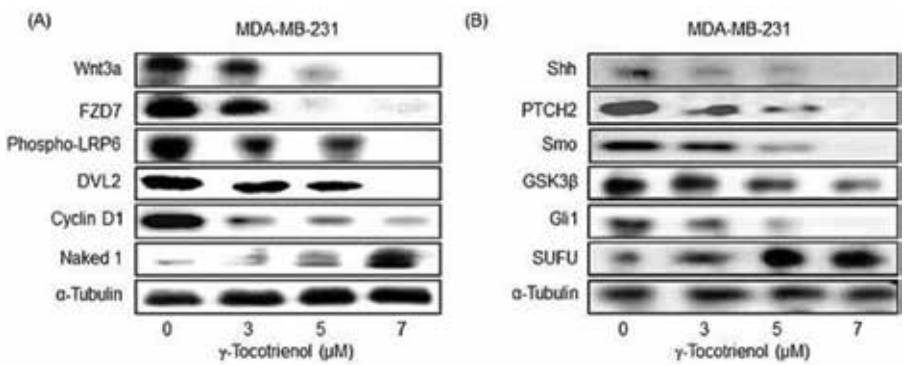

Figure 4. Western blot analysis of γ-tocotrienol effects on the canonical Wnt and Hedgehog major regulatory proteins. (A) Highly malignant MDA-MB-231 human breast cancer cells were initially seeded at density of 1×10^6 cells/100 mm dish and maintained on serum-free defined media containing different doses of γ-tocotrienol over a 4-day culture period. Following treatment exposure, whole cell lysates were prepared from MDA-MB-231 in each treatment group for consequent separation by polyacrylamide gel electrophoresis (35 μg/lane) followed by western blot analysis for the major regulatory proteins of the Wnt pathway. (B) Whole cell lysates were prepared then subjected to polyacrylamide gel electrophoresis (30 μg/lane) and western blot analysis for detection of Shh ligand, PTCH2, Smo, GSK3β, Gli1 and SUFU levels within the Hedgehog pathway.

Naked 1 (a negative regulator of Wnt pathway (**Figure 4A**). Treatment with 3–7 μM γ-tocotrienol (MDA-MB-231) induced a dose-dependent decline in Wnt3a, FZD7 receptor, phosphorylated-LRP6, DVL2, cyclin D1 levels, and a corresponding increase in Naked 1 level as compared to cells in their respective vehicle-treated control groups (**Figure 4A**). These findings indicate that γ-tocotrienol inhibition of EMT is mediated in part by a suppression of canonical Wnt signaling. Similar results were observed in T47-D breast cancer line (data not shown). Previous studies have shown that inhibition of Wnt signaling resulted in a reduction in nuclear factor erythroid 2-related factor 2 (Nrf2) activity, a transcription factor associated with the promotion of EMT [66–68]. At present, it is not known if γ-tocotrienol reversal of EMT involves a corresponding decrease in Nrf2 activity. Additional studies are required to determine if Nrf2 plays a role in the anticancer effects of γ-tocotrienol. In summary, experimental evidence strongly suggests that γ-tocotrienol therapy may provide therapeutic value in the treatment of highly malignant breast cancer that is characterized by aberrant canonical Wnt signaling.

5. Canonical Hedgehog pathway

The canonical Hedgehog pathway is characteristically over active in many forms of metastatic breast cancer and is associated with enhanced migration, invasion, stemness and self-renewal of cancer cells [69–72]. Over activity of the Hedgehog pathway is also associated with playing a role in promoting EMT [71]. The Hedgehog ligand was first discovered in the *Drosophila* fruit fly [73]. Several human Hedgehog ligands have also been identified that are involved in cell growth and controlled organ formation by insuring that the tissue reaches the accurate size and position. In the adult, this pathway normally remains quiescent. However, activation of the Hedgehog pathway may be triggered during tissue maintenance and regeneration [74]. A link between Hedgehog signaling and developmental defects was first discovered in 1996, and later that year a link between Hedgehog signaling and cancer was found when the tumor suppressor gene patched (PTCH) was discovered [67]. Soon afterwards, the Hedgehog cell service signaling transducer, smoothened (Smo), was discovered and found to have the potential to function as an oncogene. These findings lead to the development of the Hedgehog pathway inhibitor, cyclopamine, and successful clinical trials using cyclopamine and similar agents followed [75]. Hedgehog ligands are produced by three different genes. The first gene is the Indian Hedgehog (Ihh) and is found in gut, skeletal muscle, and chondrocytes [76]. The second gene is the Desert Hedgehog (Dhh) and is expressed in the testis [76].

The third gene is the Sonic Hedgehog (Shh) and is involved in many developmental processes [76]. Shh is called a morphogen since the signal of this ligand relies on its concentration [77]. The Shh is produced from zone of polarizing activity (ZPA), which is located on the posterior side of the limb bud in the embryo [78]. The Hedgehog pathway has a link with the formation of specific types of humans cancer [74]. After the transcriptional and translational process occur, this ligand is secreted as a precursor protein, and the ligand is subsequently subjected to several post translational modifications [73]. Autocatalytic cleavage then splits the ligand into two parts. One part is the signaling molecule, while the other part appears to have no function. A cholesterol molecule and palmitic acid moiety are then added to C-terminal and

N-terminal of the signaling piece, leading to an increase in its hydrophobicity, localization and binding to the receptor [73]. The canonical Hedgehog signaling pathway has several vital components which play a role in modulating signal intensity. Most of the components within the Hedgehog pathway include the Hedgehog ligand, the PTCH receptor, Smo, and the cytosolic complex and downstream effectors, which consist of suppressor of fused (SUFU) and Gli family of proteins. The Gli family is an important component of the Hedgehog pathway which is divided into three forms known as Gli1, Gli2, and Gli3. Gli transcription factors can activate the signal, have dual function to stimulate or impede the signal [79]. A number of kinases, such as GSK3β, CK1α, and protein kinase A, are known to be essential in the regulation of Hedgehog signaling [80]. The PTCH receptor of this pathway is located in the lipid raft microdomains of the plasma membrane [8].

Activation of the Hedgehog pathway can be blocked in the absence of ligand expression or a lack of mutation in PTCH and/or Smo [79]. In such cases, the inhibitory effect of PTCH on Smo is intact and Hedgehog ligand transport to the cell membrane is prevented and receptor activation and signal transduction does not occur [79]. In contrast, activation of the Hedgehog pathway will result in conditions when the Hedgehog ligand is highly expressed, and/or when mutation of PTCH and/or Smo occurs [79]. In these conditions, inhibitory effect of PTCH on Smo is absence and Smo can freely travel to the cell membrane, leading to the phosphorylation of SUFU and the transcription factor in the cytosolic complex. Once this occurs, Gli separates from the cytosolic complex proteins and then translocates into the nucleus where it promotes an increase in the Hedgehog target gene expression [79]. Recent studies have shown a direct connection between EMT and stemness of breast cancer resulting that is directly associated with the activation of the canonical Hedgehog signaling and the development of tumor recurrence and metastasis [71].

Figure 4B shows the effects of γ-tocotrienol treatment on signaling protein levels and activation within the Hedgehog pathways. Results show that the Hedgehog Shh ligand is relatively high in MDA-MB-231 breast cancer cells in the vehicle-treated control group. Similarly, PTCH2 receptor, Smo, GSK3β, and Gli1 were highly expressed, while the inhibitor for Hedgehog signaling SUFU displayed a relatively low level of expression in the vehicle-treated MDA-MB-231 human breast cancer cells (**Figure 4B**). Treatment with γ-tocotrienol induced a dose-dependent decrease in Shh ligand expression, as well as a dose-responsive reduction in PTCH2 receptor, Smo, GSK3β, and Gli1, and a corresponding increase in SUFU protein levels, as compared to MDA-MB-231 cells in the vehicle-treated control group (**Figure 4B**). These data indicated that γ-tocotrienol inhibition of EMT is also mediated by a suppression of canonical Hedgehog pathway and provides further evidence that γ-tocotrienol treatment may provide significant benefit in the treatment of metastatic breast cancer.

6. Conclusion

Results from these reports show that treatment with 0–5 μM γ-tocotrienol induced a significant dose-dependent inhibition of highly malignant MDA-AM-231 human breast cancer

cell growth after a 4-day culture period. Furthermore, canonical Wnt and Hedgehog signaling are highly expressed in these triple negative breast cancer cells, and γ-tocotrienol growth inhibitory effects are associated with a reduction in Wnt and Hedgehog signaling and regulatory proteins. Since γ-tocotrienol also induces a reversal of EMT in these cells and canonical Wnt and Hedgehog signaling pathways are involved in promoting EMT, it can be concluded that γ-tocotrienol inhibition of EMT is mediated by a corresponding reduction in canonical Wnt and Hedgehog signaling in malignant MDA-MB-231 human breast cancer cells. This hypothesis is further evidenced by the finding that γ-tocotrienol inhibition of Wnt and Hedgehog signaling and reversal of EMT is associated with a significant decrease in migration, invasion and stemness of these cells [12].

Metastasis is still the primary cause for the mortality (90%) in cancer patients with cancer. While a great deal of progress has been recently achieved in the further understanding of the molecular and cellular mechanisms involved in the metastatic process, these mechanisms are not completing understood and clinical therapies for the management and treatment of metastatic cancer remains insufficient. Expanding knowledge in gene expression, cellular behavior, and biological events of cancer cells will provide important and novel insights for the treatment of metastatic breast cancer. New biomarkers in areas, such as EMT will provide innovative chances in predictive methods of the metastatic potential of a primary tumor and a novel target for therapy. Experimental results summarized in **Figure 5** indicates some of the key targets in the treatment of EMT and metastasis and the possible role of γ-tocotrienol in the prevention and treatment of these processes.

In summary, experimental evidence demonstrates that γ-tocotrienol reversal of EMT results, at least in part, through the inhibition of canonical Wnt and Hedgehog signaling. These findings also suggest that supplemental treatment with γ-tocotrienol may be effective in providing significant benefit in the prevention and treatment of metastatic breast cancer.

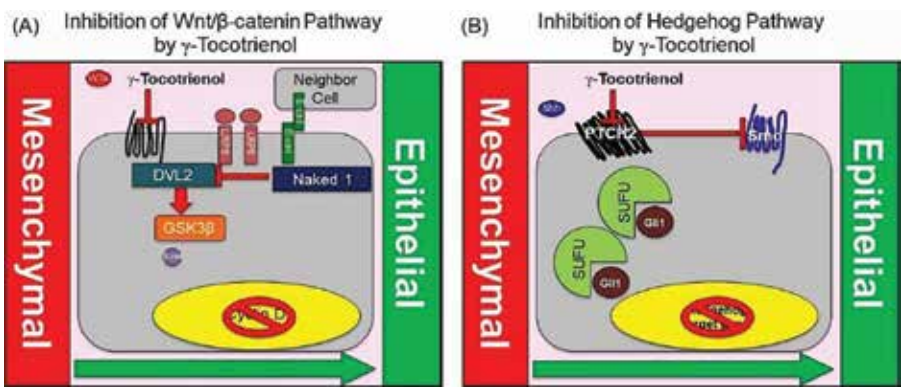

Figure 5. Schematic representation of γ-tocotrienol effects on the canonical Wnt and Hedgehog pathways and EMT. γ-Tocotrienol inhibits Wnt signaling by decreasing the expression of Wnt3a ligand, FZD7/LRP6 complex activation, DVL2 and cyclin D1 and a corresponding increase in Naked 1 level. Additionally, γ-tocotrienol inhibits Hedgehog signaling by decreasing the expression of Shh ligand, PTCH2, Smo, GSK3β, and Gli1 associated with a corresponding increase in SUFU levels. Several other cytosolic and nuclear proteins were minimized which can ultimately lead to a suppression in gene expression associated with EMT.

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Conflict of interest

There is no conflict of interest.

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In this book, *Vitamin E in Health and Disease*, the chapter by Dr Lisa Schmölz et al., The Hepatic Fate of Vitamin E, includes the hepatic metabolism of vitamin E, its storage, release, distribution, and its effects on the metabolism in great detail, as well as its effect on the prevention of diseases, in addition to its role in anti-aging. The chapter by Dr Rusu Anca Elena reports on the effect of vitamin E in patients with hemodialysis. In a similar manner, the chapter of Drs Rayan Ahmed and Paul W. Sylvester describe g-Tocotrienol, a natural isoform within the vitamin E family of compounds, which displays potent antiproliferative, apoptotic and reversal of epithelial-to-mesenchymal-transition activity against breast cancer, employing treatment doses that have little or no effect on normal cell viability. The chapter by Milka Mileva and Angel S. Galabov describes how vitamin E could be recommended as a reliable agent, indeed as a component in multiorgan flu therapy. Last, Dr Juan José Godina-Nava et al. describe the cytoprotector effect of the 120-Hz electromagnetic fields in early hepatocarcinogenesis.

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