Chapter 2

Vitamin D in Oxidative Stress and Diseases

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Additional information is available at the end of the chapter

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Abstract

The data described in this chapter consider some new information about the benefits of vitamin D_3 comparing the results obtained by the authors on the effects of vitamin D_3 during oxidative stress with other works available in the literature. In particular, vitamin D_3 can induce a concentration-dependent increase in endothelial NO production through eNOS activation consequential to the phosphorylation of p38, AKT, and ERK. Additional information obtained by the author is about the ability of vitamin D_3 to prevent the endothelial cell death through modulation of interplay between apoptosis and autophagy. This effect is obtained by inhibiting superoxide anion generation, maintaining mitochondria function and cell viability, activating survival kinases (ERK and Akt), and inducing NO production. The results also describe that vitamin D_3 causes human endothelial cell proliferation and migration in a 3-D matrix through NO-dependent mechanisms. These findings support the role of vitamin D_3 in the human angiogenic process, suggesting new applications for vitamin D_3 in tissue repair and wound healing. Finally, that the authors have demonstrated the ability of vitamin D_3 to counteract negative effects of oxidative stress in brain cells. These data suggest the potential therapeutic use of vitamin D to treat or prevent degenerative brain diseases.

Keywords: active vitamin D, extraskeletal function, oxidative stress, endothelial cells, neuronal cells

1. Introduction

Vitamin D, discovered as an essential nutrient for the prevention of rickets, is required for optimal absorption of dietary calcium and phosphate [1]. Vitamin D plays an essential role in calcium and phosphate metabolism and maintains mineral homeostasis to ensure

metabolic functions and bone mineralization. The calcium requirement of the organism is firstly satisfied by dietary calcium intake and, when this is not sufficient, by calcium mobilization from bone and renal reabsorption. Classical vitamin D–responsive tissues are bones, intestine, kidney, and parathyroid glands [1–3] and in this case, vitamin D induces skeletal effects. In addition many other organs respond to vitamin D, such as prostate, breast, immune cells, skeletal muscle, cardiac tissue, parathyroid glands, skin, and brain; all these organs express vitamin D receptor (VDR) and the enzyme 1α-hydroxylase and vitamin D exert extraskeletal effects [4]. Vitamin D exerts its activity through two mechanisms: the hormone signaling, in which the biologically active form reaches target cells through the bloodstream; and the autocrine/paracrine signaling, in which locally produced-vitamin D_3 affects the surrounding cells [5].

A variety of research results from recent years have shown that vitamin D in its hormonally active form, 1α,25-dihydroxyvitamin D [1α,25(OH)2D; calcitriol] is not only a regulator of calcium and phosphate homeostasis, but has numerous extraskeletal effects. These include the significant impact of the vitamin D hormone on the cardiovascular system, central nervous system, endocrine system, and immune system as well as on cell differentiation and cell growth [4]. The term "vitamin D" refers to two different forms playing an important role in humans: vitamin D2 (ergocalciferol), which is made in some plants but largely in fungi [6], and vitamin D_3 (cholecalciferol), which is made by human skin when exposed to sunlight via the UV irradiation of 7-dehydrocholesterol [6]. This photo-production is influenced by several factors including ethnicity (skin pigmentation), UV exposure (latitude, season, use of sunscreens, and clothing), and age. All these factors may reduce the ability to synthesize the bioactive form of vitamin D. Moreover, low outdoor activity and unhealthy lifestyle habits are also cause for a scarce vitamin D_3 production. Conversely, excessive exposure to sunlight degrades pre-vitamin D and vitamin D_3 into inactive photoproducts. Many organs participate in the synthesis of the bioactive form of vitamin D [7, 8]. Vitamin D_3 needs two hydroxylation steps in liver and kidney for its activation. The final product, hormonally active 1α,25-dihydroxyvitamin D_3 (calcitriol), arrives via the circulation to its target tissues and acts in a genomic or nongenomic manner [9].

All genomic actions of vitamin D are mediated by the VDR. VDR is a transcription factor and a member of the steroid hormone nuclear receptor family. VDR binding to its vitamin D response elements (VDREs) causes the recruitment of coregulatory complexes required for its genomic activity. These complexes can be both gene and cell specific, enabling the selectivity of vitamin D_3 action from cell type to cell type [8]. The profile of VDR binding sites and genes activated by vitamin D_3 varies from cell to cell with some albeit far from total overlap especially when results with different time durations of vitamin D_3 exposure are compared [10]. Moreover, these VDR binding sites can be anywhere in the genome, often many thousands of base pairs away from the gene to be regulated. Generally, these sites are associated with binding sites for other transcription factors [11, 12].

Vitamin D_3 is also able to activate very rapid nongenomic mechanisms [8], lasting from seconds to 10 minutes [13]. This mechanism involves signal transduction pathways including activation of adenylyl cyclase-cAMP-protein kinase A and phospholipase C-diacylglycerol-inositol

(1,4,5)-trisphosphate-protein kinase C signal transduction pathways [14]. Particularly, the second messengers Raf (rapidly accelerated fibrosarcoma)/MAPK play an important role because they may engage in cross-talk with the nucleus to modulate gene expression [13]. This firstly identified nongenomic activation is associated with the rapid stimulation of intestinal calcium transport called "transcaltachia" [15]. Successively, this effect was identified in the chondrocytes of the bone growth plate [15] and in keratinocytes of the skin [16]. Identification of the receptor for vitamin D_3 has focused on the VDR itself albeit in a different configuration to identify agonists able to induce nongenomic effects [17]. Interaction between vitamin D_3 and membrane-associated rapid response steroid binding protein (MARRS) has been studied as well. These receptors are located in the membrane within caveolae/lipid rafts [18] where they are poised to activate kinases, phosphatases, and ion channels.

The aim of this paper is to illustrate the most recent findings about the role of vitamin D_3 as a potent regulator of oxidative pathways.

2. Relevant extraskeletal applications

In a recent study, Berridge suggests the idea that vitamin D plays a crucial activity in keeping the integrity of cell signaling pathways coining the term of "custodian of phenotypic stability" [19]. The hypothesis supported is that a loss of this integrity could be explained by vitamin D deficiency, and this represents a risk factor for different diseases. This role assigned to vitamin D may depend on the regulation of the expression of nuclear factors involved both in physiological control of ROS and in calcium signaling [19]. The hormonally active form of vitamin D, $1\alpha25(OH)2D_3$, influences the expression of various genes, whose products not only are involved in the control of calcium and phosphate homeostasis, but are also able to interact with a wide range of organs and target tissues different from those involved in calcium metabolism [20]. Most experimental studies and meta-analysis showing a large amount of information concluded that vitamin D supplementation is associated with a decrease in total mortality [21]. In effect, vitamin D_3 is involved in a fine balance among organs and tissues that contributes to the maintenance of the homeostasis of the human organism. For example, an important function of vitamin D_3 is to maintain normal parathyroid status, preventing proliferation of parathyroid gland cells. Patients with renal failure-dependent vitamin D_3 deficiency, in presence of adequate calcium levels, may be affected by parathyroid hypertrophy with consequent secondary hyperparathyroidism [22]. Moreover, vitamin D_3 acts as a negative endocrine regulator of the renin-angiotensin system. It enters the bloodstream and downregulates renin production and stimulates insulin secretion from β-cells of the pancreatic islets [1, 3]. Vitamin D_3 is thought to possess positive effects on muscles, affecting the calcium handling in the cells and promoting de novo protein synthesis. Vitamin D_3 maintains calcium balance in cultured muscle cells initially via inositol triphosphate induced-rapid ion release from the sarcoplasmic reticulum and then through ion channels that allows calcium influx from the extracellular compartment. Recently, vitamin D_3 deficiency has been connected with sarcopenia (age-related loss of muscle mass) mainly of type II skeletal muscle fibers. Vitamin D supplementation reduces incidence of fractures, actually decreasing the number of falling

[4, 7]. Sarcopenia was initially defined as "disease of the elderly" and a degenerative consequence of ageing, but successively it can be also diagnosed at younger age [23]. Except for ageing, risk factors for sarcopenia include female gender, low physical activity, low protein intake, and vitamin D deficiency in both older and younger populations [23, 24]. Vitamin D levels decline with age and cutaneous vitamin D levels are up to four times lower in older than in younger individuals [25, 26]. Low vitamin D levels result in atrophy predominantly of the type 2 muscle fibers and it has been associated with an increase in sarcopenia [27]. Finally, the serum level of calcitriol is different in younger and elder people and it is related to incidence of sarcopenia.

Vitamin D_3 is a potent immunomodulator. For example, after a skin lesion, keratinocytes that compose the mucocutaneous barrier upregulate VDR and 1α-hydroxylase expression in order to improve immune defenses. Monocytes and macrophages act in the same way after a Mycobacterium tuberculosis infection or exposure to lipopolysaccharides. An increased cathelicidin and α-defensin 2 production results in all cases and these two proteins could display their antimicrobial effects, promoting innate immunity. It has been hypothesized that monocytes or macrophages may also release self-produced-vitamin D_3 to act locally on activated T and B lymphocytes, which are able to regulate cytokine and immunoglobulin synthesis, respectively [3, 28]. The large number of effects of vitamin D_3 regulating the immune system plays a very important role in fighting infectious diseases [29]. Vitamin D_3 enhances the innate immunity against various infections [30], especially tuberculosis, influenza, and viral upper respiratory tract infections [29]. Evidence exists about the potential antimicrobial activity of vitamin D and its deficiency has deleterious effects on general well-being and longevity. Vitamin D reduces the risk of infection in different manner: by modulating production of antimicrobial peptides, by regulating local immune and immunoinflammatory response, and by enhancing clearance of invading organisms. In addition, vitamin D constitutes an inexpensive prophylactic option and possibly a therapeutic product either by itself or as a synergic agent to the traditional drugs [31]. Ecological studies have shown that the prevalence of certain autoimmune diseases was associated with latitude, suggesting a potential role of sunlight exposure, and consequently vitamin D_3 production, on the pathogenesis of type 1 diabetes mellitus, multiple sclerosis and Crohn's disease. The increased prevalence at higher latitudes has been shown for multiple sclerosis (MS), inflammatory bowel disease, rheumatoid arthritis, and type 1 diabetes [32–36]. In children with asthma, vitamin D levels also seem to correlate positively with asthma control and lung function, and inversely with corticosteroid use [37–39].

Many experimental data show that calcitriol stimulates apoptosis and differentiation and inhibits angiogenesis and proliferation in tumor cells. Numerous association studies suggest that serum vitamin D levels are inversely associated with the risk of many types of cancer. Furthermore, in some studies of patients with cancer, an association between low vitamin D levels and poor prognosis has been observed [40–42]. In breast, colon, and prostate cancer, vitamin D_3-VDR complex could arrest cell cycle in the G1-G0 transition, inducing p21 and p27 synthesis and/or stabilization, blocking cell growth and promoting cell differentiation. In TGF-α/EGFR-dependent tumor, vitamin D_3 can inhibit the growth inducing the recruitment of the

activated receptor into early endosomes, and reducing growth signaling [21]. In monocytes and osteoblast culture, it seems that the biologically active form of vitamin D_3 could enhance the expression of a suppressor of the oncogenic cyclin D1 and C/EBPβ. Moreover, vitamin D_3 is able to regulate apoptosis. It could induce this process, like in breast cancer cells for example, where it may modulate Bcl2 and Bax contents. However, it could also show antiapoptotic effects, essential in normal tissue development and function. For instance, it protects keratinocytes from UV-irradiation or chemotherapy-induced apoptosis and melanocytes from TNF-α-mediated apoptosis. In normal keratinocytes, vitamin D_3 induces cell differentiation and maintains a calcium gradient necessary for the integrity of the permeability barrier of the skin. Vitamin D_3 represses tumor invasion and metastasis reducing matrix metalloproteinase activity (MMP) and enhancing the expression of molecules with adhesive properties such as E-cadherin [43]. However, calcitriol is a multifunctional steroid hormone able to regulate signaling pathways related to cancer development and progression. In preclinical studies, it was shown that vitamin D can promote cell differentiation and inhibit proliferation, angiogenesis, and cell migration. Inconsistent results are found in epidemiological studies and in early trials regarding clinical effects of vitamin D supplementation and cancer in terms of prevention and impact in cancer-related mortality [44]. Vitamin D also exerts its effects on cancer through nongenomic factors, modulating inflammatory cytokine expression. This is a common element for cancer and neurodegeneration.

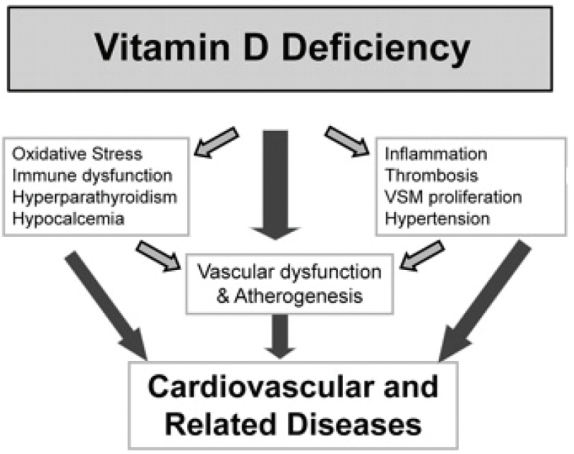

Figure 1. Vitamin D and cardiovascular risk (adapted from [48]).

Another important feature of vitamin D is the mitochondrial detoxification, well known as antioxidant activity. Especially, vitamin D and polyphenols, seem to be promising therapeutic tools for inhibiting radical oxygen species (ROS) formation and arresting cytokine-mediated inflammation [45].

Vitamin D status varies with age [46]. Serum levels of calcitriol are normal from 75 nM, insufficient from 50 to 75 nM, and deficient if less than 50 nM [47]. These serum concentrations depend on sunlight exposure, dietary intake, capacity of the skin, and influence various human diseases (cardiovascular and neurological), as reported in this manuscript (**Figures 1** and **2**).

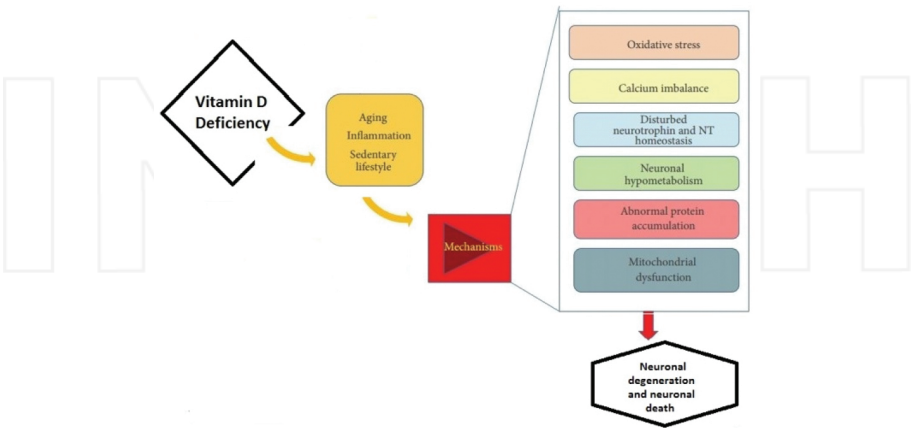

Figure 2. Vitamin D and neurological diseases (adapted from [49]).

2.1. Cardiovascular and cerebral risk

In humans, the relationship among low levels of vitamin D_3, hypercalcemia, osteoporosis, vascular calcification, and cardiovascular diseases has been extensively studied. Vitamin D_3 deficiency is significantly associated with cardiomyopathy and increased risk of cardiovascular disease with consequent mortality in humans [8]. The prospective Intermountain Heart Collaborative Study revealed that vitamin D_3 blood levels below 15 ng/mL compared to vitamin D_3 above 30 ng/mL are associated with significant increases in the prevalence of type 2 diabetes mellitus, hypertension, hyperlipidemia, and peripheral vascular disease, coronary artery disease, myocardial infarction, heart failure, and stroke, as well as with incident death, heart failure, coronary artery disease/myocardial infarction, and stroke [50].

Several data are available from studies in mouse animal model. Heart-VDR null mice show myocardial hypertrophy, while VDR and CYP27B1 null mice develop hypertension as well and display increased production of renin, contributing probably to early atherosclerosis onset [51]. Vitamin D_3 may protect against atherosclerosis inhibiting macrophage cholesterol uptake and foam cell formation, and reducing vascular smooth muscle cell proliferation and expression of adhesion molecules in endothelial cells. Recently, elevated levels of PTH emerged as a possible risk factor for cardiovascular diseases since they could promote development of hypertension; in addition, hyperparathyroidism could cause heart hypercontractility and calcification of the myocardium. Vitamin D_3 could overcome these processes, promoting, for example, synthesis of anti-inflammatory cytokines [4]. Severe vitamin D_3 deficiency in humans

is associated with cardiomyopathy [52], and in a number of large epidemiologic studies, the association of increased cardiovascular disease risk with low vitamin D_3 blood levels has been found [53]. Obesity, a condition associated with cardiovascular disease [54], is related with a low vitamin D_3 status due to a sequestration and volumetric dilution of the lipophilic vitamin D_3 in the fat tissue [3, 55, 56]. Many studies suggest a biological beneficial effect of vitamin D_3 on cardiovascular risk factors and cardiovascular health. For example, in a recent study performed on Framingham Offspring Heart Study participants [57], vitamin D_3 deficiency was associated with increased cardiovascular risk, above and beyond established cardiovascular risk factors. In another study, performed on elderly subjects, an association between low serum vitamin D_3 levels and high arterial blood pressure was found [58]. In addition, vitamin D_3 insufficiency/deficiency is associated with myocardial infarction, congestive heart failure, and calcific aortic stenosis, which can lead to the massive vascular calcification seen in chronic kidney disease. Recent clinical studies demonstrated that vitamin D_3 may cause regression of cardiac hypertrophy, reduces cardiovascular morbidity, and mortality in patients who frequently suffer from atherosclerosis [59, 60] and it may improve the cardiac structure and function [61].

The VDR is present in endothelium, vascular smooth muscle, and cardiomyocytes [55, 57] and may protect against atherosclerosis through the inhibition of macrophage cholesterol uptake and foam cell formation, the reduction of vascular smooth muscle cell proliferation, and the decrease of expression of adhesion molecules in endothelial cells [55] and through inhibition of cytokine release from lymphocytes [57]. Several meta-analyses indicate an inverse association between vitamin D_3 status and hypertension, and the antihypertensive effects were associated with a rate in vitamin D_3 levels after dietary supplementation or UVB exposure [62–66]. Mechanistically, this effect could be partly mediated by vitamin D_3's capability to suppress the levels of PTH, which is the cause of arrhythmias, myocardial hypertrophy and increasing blood pressure [67], and the levels of renin [67, 68]. As far as endothelial effects are concerned, it has been demonstrated that vitamin D_3 modulates vascular tone by means of a reduction in calcium influx into the endothelial cells followed by a decrease in endothelium-derived contracting factors production. Vitamin D_3 exerts its physiological effects acting on VDR through both genomic and nongenomic mechanisms, regulating cellular proliferation, differentiation, apoptosis, and angiogenesis in local tissue [69–71]. In particular, many vascular effects, such as increased expression of Ca-ATPase, induction of contractile protein synthesis, and, hence, increased vascular resistance have been reported. Vitamin D_3 is a modulator of vascular wall growth as well [72] and induces a decrease in the expression and/or secretion of proinflammatory and proatherosclerotic factors in the endothelium [73]. The role of endothelium as a target of vitamin D_3 is demonstrated by the study published by Zehnder et al. [74], in which the expression of mRNA and protein for lα-hydroxylase in human endothelium was shown for the first time. Altogether, these findings demonstrated the direct effects of vitamin D_3 on endothelial function, whose alteration plays an important role in the development of atherosclerosis. Endothelial cells are capable of synthesizing vitamin D_3 as results from the expression of mRNA and protein for the enzyme 25(OH)D_3-1α-hydroxylase [75]. Moreover, studies elsewhere have demonstrated the presence of intracellular vitamin D_3 receptors within endothelial cells (VDR) [76]. Endothelial cells can synthesize vitamin D_3 due to the expression

of the key biosynthetic enzyme 25 (OH)D_3-1a-hydroxylase [74]. Because of the presence of both vitamin D_3 and VDR in endothelial cells and the key role of nitric oxide (NO) in the endothelial physiology, it is possible to hypothesize an interaction between vitamin D_3 and NO capable of influencing proliferation and migration of endothelial cells [75]. Although the relationship among vitamin D_3, endothelium, and cardiovascular disease is well established, little is known about the effect of vitamin D_3 on endothelial NO production. In addition, several recent studies have postulated anti-inflammatory, immunomodulatory, and neuroprotective functions for vitamin D_3. A meta-analysis examining the association between vitamin D_3 status and the risk of cerebrovascular events, including more than 1200 stroke cases, found that the pooled relative risk for stroke was 52% higher when comparing 25(OH)D levels ≤12.4 ng/mL with 25(OH)D levels >18.8 ng/mL [77].

As concerned to cerebral effects, the observed widespread distribution of 1α-hydroxylase and the nuclear VDR in both neurons and glial cells suggest that vitamin D_3 may have autocrine and paracrine properties in the brain [78, 79]. VDR is highly expressed in multiple brain regions [80] in the animal [81] and human [82] brain, particularly in the pontine-midbrain area, cerebellum, thalamus, hypothalamus, basal ganglia, hippocampus, olfactory system, and the temporal, orbital, and cingulate areas of brain cortex [83]. Mounting evidence indicates that vitamin D_3 and its receptors play an important role in the brain, ranging from neuroprotection to immunomodulation [84]; cells proliferation and differentiation [85], and plays an important role both in developing [86] and adult brain [80]. Vitamin D_3 can exert these effects since it is able to cross the blood-brain barrier and can bind to VDR within the brain [87, 88]. Upon binding, VDR undergoes a conformational change to form a complex with a retinoid X receptor that controls genic expression [89, 90]. More recent papers demonstrate that gestational vitamin D_3 deficiency induces long-lasting alterations in the brain structure, including changes in volume, cell proliferation and reduction in the expression of nerve growth factors (NGF), glia-derived neurotrophic factor (GDNF) and neurotrophins 3 and 4 [80, 83, 91]. Moreover, vitamin D_3 protects neurons against NMDA, glutamate, 6-hydroxydopamine, and reactive oxygen species [92, 93]. It has been hypothesized that vitamin D_3 exerts its neuroprotective effects via the modulation of neuronal Ca^{2+} homeostasis, in particular through the downregulation of the L-type voltage-sensitive Ca^{2+} channel in hippocampal neurons against excitotoxic insults [80], accompanied by an increase in VDR density. Vitamin D_3 is able to inhibit proinflammatory cytokine and NOS [94] typically increased during various insults or disorders, such as ischemia and reperfusion, Alzheimer's disease, Parkinson's disease, multiple sclerosis, and experimental autoimmune encephalomyelitis. Early vitamin D_3 deficiency may be considered as a risk factor for a number of neurological disorders including schizophrenia, autism [88, 95], multiple sclerosis, Parkinson's disease, and stroke [77, 89]. Vitamin D_3 deficiency is associated with reduced cognitive function, which is an important issue for stroke patients [96]. Moreover, in the rat model of stroke, vitamin D_3 supplementation has been found to reduce brain damage [97] and consequent seizures [77] and oxidative stress [98]. For this reason, it should be very important to study the role of vitamin D_3 in counteracting negative effects of oxidative stress in vitro brain, also in association with stem cell. So, vitamin D_3 may be considered as a potential drug for the treatment of neurodegenerative disorders.

3. Oxidative stress and injury

Xiang et al. [99] showed the ability of vitamin D_3 to stimulate endothelial cell proliferation and to inhibit apoptosis by increasing endothelial nitric oxide synthase (eNOS) expression and nitric oxide (NO) production. NO plays a key role in cardiovascular physiology [100], and its production in the heart by eNOS phosphorylation represents an important regulating factor of both myocardial perfusion after ischemia and myocardial contractility. This has important effects on cardiac cell functions such as oxygen consumption, hypertrophic remodeling, apoptosis, and myocardial regeneration [101–103]. The discovery of NO as a signaling molecule in the cardiovascular system was published in 1998 [104] and it is now recognized as an endogenous vasodilator and an antioxidant factor able to regulate the vascular endothelium supporting its anticoagulant and antithrombogenic capacities, maintaining vascular tone and preventing vascular smooth cell proliferation [105]. NO acts as a signaling molecule by binding to ferrous heme within metalloproteins (such as soluble guanylate cyclase, sGC, cytochrome c oxidase, and hemoglobin). The most important action mechanism of NO is the interaction with heme of the sGC in smooth muscle cells adjacent to the endothelium, catalyzing the conversion of guanosine triphosphate (GTP) to cGMP [106]. cGMP-dependent protein kinases promote the opening of calcium-dependent potassium channel and determine hyperpolarization of the cell membrane of smooth muscle, thus inhibiting cytosolic calcium influx and promoting cell relaxation and vasodilation. Alternatively, NO can determine molecular modifications. Since it is a highly diffusible molecule in tissues and physiological fluids with a very short half-life (few seconds), it rapidly reacts with superoxide anion (O^{2-}) to form peroxynitrite ($ONOO^-$). $ONOO^-$ oxidizes DNA, proteins, lipids, and BH4, uncouples eNOS and limits NO production. $ONOO^-$ directly influences the dilatory capabilities of the arteries and disrupts NO-induced sGC-mediated signal transduction [107]. Nevertheless, NO could be transported by protein carriers or stored locally and cause remote and long-lasting effects in the cardiovascular system [108, 109]. In addition NO is able to enhance endothelial cell survival, proliferation, and migration [110, 111]. Furthermore, NO signaling depends on its own concentration. Thomas and colleagues demonstrated in MCF7 cells (human breast adenocarcinoma cell line) that NO levels ranging from 10 to 30 nM lead to extracellular signal-regulated kinases (ERK) phosphorylation. This effect is due to a cGMP-dependent process, mediating proliferative and protective effects. It has been demonstrated that low NO levels, below 1 nM, are sufficient to obtain this response in endothelial cells. When the NO concentration is between 30 and 60 nM, Akt phosphorylation occurs. This antiapoptotic mechanism depends on Bad and caspase-9 phosphorylation. Hypoxia-inducible factor-1α (HIF-1α) is stabilized when NO is at a high concentration (about 100 nM) and protects against tissue injury, while at 400 nM p53 is posttranslationally modified, resulting in growth arrest or apoptosis. Signal transduction cascades react accordingly to NO not only in terms of concentration but also in terms of duration of exposure with different threshold sensitivities, resulting in distinct phenotypic responses. Indeed, HIF-1α is an immediate but transient responder and protein disappears when the NO concentration falls under the minimum threshold. On the contrary, p53 has a delayed response that takes several hours to occur but is sustained long after NO

exposure [112]. Despite the differences in clinical manifestations and neuronal vulnerability, the pathological processes appear similar in neurodegenerative pathways as well.

Cell death and neurodegenerative conditions have been linked to oxidative stress and imbalance between generation of free radicals and antioxidant defenses. Multiple sclerosis, stroke, and neurodegenerative diseases have been associated with the reactive oxygen species and nitric oxide [113]. Recent findings about the production of the reactive oxygen and nitrogen species by NADPH oxidases and nitric oxide synthases and the essential role of glutathione (γ-glutamyl-l-cysteinylglycine) in redox homeostasis demonstrate the importance of these substances in neuronal degeneration. Redox signaling has a profound impact on two transcription factors that modulate microglial fate, nuclear factor kappa-light-chain-enhancer of activated B cells, and nuclear factor (erythroid-derived 2)-like 2, master regulators of the pro-inflammatory and antioxidant responses of microglia, respectively. The relevance of these proteins in the modulation of microglial activity and the interplay between microglia and oxidation has been described. Finally, the relevance of ROS in altering blood-brain barrier permeability is reported. ROS originates in the mitochondrial electron transport chain (mETC) or are produced by NADPH oxidases (NOX) by an incomplete one-electron reduction of oxygen. Three major ROS exist: superoxide anion (O^{2-}), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH). In cellular respiration, most of O_2 generates H_2O_2 through enzymes of the superoxide dismutase (SOD) family, enclosed copper-zinc superoxide dismutase (SOD1 or Cu, Zn-SOD) present in the intermembrane space and in the cytosol, manganese superoxide dismutase (SOD2 or Mn-SOD) present in the mitochondria matrix, and SOD_3 present in the extracellular matrix. Hydrogen peroxide could subsequently be converted to the hydroxyl radical by ferrous, or a final reducing step could convert it to water by catalase, glutathione peroxidase, and peroxyredoxin III activity.

In endothelial cells, ROS into the cytosol could stimulate further ROS production by NOX2 through redox-sensing protein kinase C (PKC) isoforms and Src family kinases activation [114, 115]. Recent examples of the importance of these findings about ROS and NO in the onset or progression of neurodegenerative diseases are also showed [116]. Three NO-synthase isoforms could produce NO: (1) NO-synthase I (or neuronal, nNOS, NOS-1) expressed in neurons and skeletal and smooth muscle; (2) NO-synthase II (or inducible, iNOS, NOS-2) in immune cells; and (3) NO-synthase III (or endothelial, eNOS, NOS-3) in cardiomyocytes and platelets and, predominantly, in endothelial cells [109, 117]. NOS isoforms generate NO at different rates: eNOS and nNOS produce a low concentration of NO, leading to physiological processes regulation, while iNOS generates a high NO concentration in response to inflammatory stimuli, such as in activated macrophages, to establish cytotoxic effects and antipathogen reactions [118]. eNOS is a Ca^{2+}/calmodulin dependent enzyme and contains an N-terminal oxygenase and a C-terminal reductase domain, linked by a calmodulin binding sequence. It works as a homodimer to synthesize NO and L-citrulline from L-arginine and oxygen. In the presence of several cofactors, such as nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), heme, tetrahydrobiopterin (BH4), and zinc, it catalyzes a five-electron oxidation of one of the guanidino nitrogens of L-arginine. Since it is a gas, NO then diffuses to the vascular smooth muscle cells and reacts with

sGC, leading to cGMP-mediated vasodilatation. In endothelial cells, after enzymatic acylation, myristoylation and reversible palmitoylation, eNOS is predominantly located in the plasma membrane and in the Golgi apparatus associated with caveolin-1 located in caveolae, whose binding inhibits the activity of the enzyme [108, 109]. Two mechanisms leading to eNOS activation and involving G protein coupled receptors and heterotrimeric G protein have been explained: intracellular Ca^{2+} mobilization through phospholipase C (PLC) pathway, and phosphatidylinositol-3-kinase (PI3K)/Akt pathway [119, 120]. An increase in intracellular calcium concentration, indeed, is a critical determinant that causes eNOS dissociation from caveolin-1, leading to the activation of the enzyme. Moreover, serine, threonine, and tyrosine phosphorylation regulate eNOS activity, either enhancing or suppressing it, depending on the residue and the domain that are involved. Several eNOS agonists such as VEGF, bradykinin, and adenosine triphosphate (ATP) promote the activation of phosphorylation. eNOS expression and activation is also sensitive to shear stress caused by blood flow; low shear stress determines lower NO production, increasing atherosclerotic plaque formation [108, 109]. Endothelial dysfunction should be considered as endothelial activation from a quiescent phenotype and it is identified as an initial step in the development of CVD. This is characterized by reduced NO bioavailability that predisposes to vasoconstriction and thrombosis [121]. Several factors can contribute to this process, such as decreased eNOS expression, presence of eNOS antagonist, low L-arginine or BH4 concentration, increased NO degradation or ROS production, exposure to inflammatory cytokines and growth factors [95]. Indeed, the fundamental change is a switch in the signaling from an NO-mediated silencing of cellular processes toward activation by redox signals. The generation of hydrogen peroxide that could react with cysteine groups in proteins alters their function, determining transcriptional factor phosphorylation, induction of nuclear chromatin remodeling, and protease activation. eNOS, normally involved in the maintenance of the quiescent state of the endothelium, could be uncoupled, in the case of substrate L-arginine deficiency. Consequently, superoxide formation occurs if the key cofactor tetrahydrobiopterin is not present, or hydrogen peroxide production is established. The mitochondria are a source of ROS during hypoxia or conditions of increased substrate delivery, such as in obesity-related metabolic disorders or type II diabetes, which are characterized by hyperglycemia and increased circulating free fatty acids. The interaction between ROS and NO results in further endothelial activation and inflammation [122].

4. Protective effects of vitamin D

Although the relationship among vitamin D_3, endothelium, and cardiovascular disease is well established, until recently little was known about the effect of vitamin D_3 on endothelial NO production. NO plays a key role in cardiovascular physiology [100], and its production in the heart by eNOS phosphorylation represents an important regulator of both myocardial perfusion after ischemia and myocardial contractility and has important effects on cardiac cell functions such as oxygen consumption, hypertrophic remodeling, apoptosis, and myocardial regeneration [101–103]. In addition, NO is able to enhance endothelial cells survival, proliferation, and migration [123]. In recent works, cells proliferation has been evaluated in low serum

conditions to synchronize cell culture after 24 or 48 h in starvation medium in the presence or absence of vitamin D (1–10–100 nM) for porcine aortic endothelial cells (PAE) [123] and human umbilical vein endothelial cells (HUVEC), respectively [124, 125]. Vitamin D_3 induces a significant dose-dependent increase in cell growth at all concentrations tested. The maximal effect is reached by stimulating PAE cells with 10 nM vitamin D_3, while at the highest concentration tested (100 nM) the vitamin D_3 effect on cell proliferation is less potent ($p < 0.05$). The observed cellular density is almost doubled compared to control samples for PAE cells (690 ± 210 cells/mm^2 vs 354 ± 84 cells/mm^2, $p < 0.01$) and HUVEC. Proliferation assays were also performed in the presence of L-NAME, the arginine analog inhibiting NO synthesis, in order to evaluate NO involvement. In these conditions, vitamin D_3 is no more able to induce cell proliferation, in all cell types. The presence of L-NAME does not alter control cell proliferation, whereas it completely reverts vitamin D-induced cell proliferation. The effective involvement of VDR in these effects has been demonstrated as well. HUVEC were stimulated with the VDR ligand ZK159222. Under these conditions, a significant decrease in vitamin D–induced cell proliferation has been observed, confirming the role of VDR in the effects mediated by vitamin D_3. In addition, an increase in cell mitosis has been observed only in vitamin D_3 treated specimens and not in samples with L-NAME. Cell migration has been evaluated in a three-dimensional model by an anionic hydrogel made of gelatin and polyglutamic acid, which has previously been described as a good substrate for cell growth [126, 127] and was lean on 70% confluent cell monolayers. PAE migration, evaluated by counting the cells migrated in the 3-D matrix for 7 days, increases significantly only in the presence of 100 nM vitamin D_3 ($p < 0.05$). HUVEC migration increases significantly only in the presence of 10 nM and 100 nM vitamin D_3 ($p < 0.05$ and $p < 0.01$ respectively). L-NAME treatment does not affect control cell migration, while it significantly reduces vitamin D-induced hydrogel invasion and ZK159222 reduces the vitamin D-induced migratory effect. Extracellular matrix degradation is one of the main steps in cell migration; for this reason, vitamin D affects on MMP-2 expression in the conditioned medium of cells migrating into the 3-D hydrogel matrix after 7 days has been evaluated by gelatin zymography. Vitamin D_3 addition to PAE culture medium increases MMP-2 production in a dose-dependent manner. In HUVEC, gelatin zymography demonstrates a statistically significant increase in MMP-2 activity in all the vitamin D-treated samples. This effect is more evident in samples stimulated with 100 nM vitamin D_3 ($p < 0.01$). The increase in MMP-2 expression appears to be NO dependent, as L-NAME treatment totally abrogates vitamin D_3 effects on MMP-2 expression, according to the above described results for cell migration. In this context it is very important verify the effect of vitamin D_3 in HUVEC on NO production and the intracellular pathways activated by vitamin D_3 leading to eNOS activation.

Vitamin D_3 is able to stimulate NO production in HUVEC in a dose-dependent manner accompanied by a significant increase in the level of phosphorylation of intracellular kinases. The administration of vitamin D_3 induced the highest production of NO, acutely increased the phosphorylation of eNOS, p38, AKT, and ERK, which are known to be involved in the intracellular signaling leading to NO production [128]. The effects were prevented by L-NAME or specific protein kinase inhibitors such as SB203580, wortmannin, and UO126 related to p38, AKT, and ERK in endothelial cells [129]. Another important finding is the demonstration of the involvement of VDR in vitamin D-induced endothelial NO production [76]. This fact is

shown by VDR antagonist ZK159222 and by VDR agonist ZK191784. These data on the role of VDR ligands on endothelial NO production add new information on the possible therapeutic role of these substances. VDR plays an important role in modulating cardiovascular function and early interventional studies in humans demonstrated that VDR analogs therapy seems to be more effective than native vitamin D supplementation in modulating cardiovascular disease risk factors [130]. It is noteworthy that vitamin D_3 response occurs within seconds and, for this reason, it appears to be a nongenomic effect. This fact about the role of the concentration of vitamin D_3, is also important to explain same data reported in the literature about the ability of vitamin D_3 to prevent myocardial ischemia in patients with a decreased serum vitamin D_3 concentration as reported in a case-control study in 1990 on 179 patients [131] in which the odds of having a myocardial infarction increased along. In another study, Giovannucci et al. [132] demonstrates that men with vitamin D_3 deficiency had a higher risk of myocardial infarction compared with those with normal vitamin D_3 concentration.

Recent investigations on cardiac myocytes showed that ROS produced by mitochondrial and oxidative stress can cause multiple changes in the cell structure and the function that are associated with a failing heart [133] and apoptosis [134] or autophagy [135]. On the other hand, ROS, along with NO, show antiapoptotic effects involving several signaling pathways; for example, not only activates proapoptotic signals [135] but also potently induces autophagy [136]. The functional role of autophagy during ischemia/reperfusion is complex, because its pathophysiological functions depend on the severity and duration of ischemia (hypoxia) and the consequent tissue damage during reperfusion in the heart. The level of autophagy may determine whether autophagy itself is protective or detrimental in response to ischemia/reperfusion injury in the heart [137]. Vitamin D_3 is able to induce autophagy in various human cell types. The signaling pathways regulated by vitamin D_3 include, for example, Bcl-2, beclin 1, and mammalian target of rapamycin (mTOR) [138]. The interplay between autophagic and apoptotic pathways is a crucial point to determine the initiation of programmed cell death and whether the members of the beclin 1 and Bcl-2 family were involved. Beclin 1 directly interacts not only with Bcl-2 but also with other antiapoptotic Bcl-2 family proteins such as Bcl-xl, and Bcl-2 was able to inhibit the beclin 1-dependent autophagy [139]. It has been clearly demonstrated that administration of vitamin D_3 to endothelial cells before induction of an oxidative stress can improve cell viability [20]. The mechanisms involved include the prevention of free oxygen radical release and the modulation of the interplay between apoptosis and autophagy. These effects were also accompanied by NO production and preservation of mitochondrial function. HUVEC cultures received an oxidative stress by means of H_2O_2. This method is widely used to reproduce a cellular damage similar to what occurs in myocardial ischemia/reperfusion injury [20]. In light of these data, a cardioprotective role of vitamin D_3 against the ischemic injury can be hypothesized. As observed in NO production and MTT tests, the effect induced by VDR agonist ZK191784 is greater than the one observed after vitamin D_3 alone. Moreover, the combined administration of the two VDR agonists (Vit D and ZK191784) induced an amplified effect. These beneficial effects were observed when VDR agonists were administered before the induction of oxidative stress. This fact supports the hypothesis that vitamin D_3 is able to counteract the negative effects of the oxidant event on endothelial cells, increasing cell viability. In addition, NO release induced by vitamin D_3 during oxidative stress

is able to protect cells from death. This result is demonstrated by the observation that the rate of NO production was below 2¼M/s. This threshold prevents the opening of the mitochondrial transition pore and the release of cytochrome C and avoids mitochondrial collapse leading to cell death [140]. The antiapoptotic effects of NO and ROS involved several signaling pathways, and the interplay between autophagic and apoptotic pathways is a crucial point to determine the starting of programmed cell death. Another important finding is that pretreatment with vitamin D_3, alone or in combination with ZK191784, is able to reduce the apoptosis-related gene expression (Bax, caspase-3, caspase-9, caspase-8, and cytochrome C), involving both intrinsic and extrinsic pathways. These findings were confirmed by immunohistochemistry analysis of annexin V and TUNEL assay in which we observed a signal reduction. At the same time, activation of pro-autophagic beclin 1 and the phosphorylation of ERK1/2 and Akt, members of the reperfusion injury salvage kinase pathway, have been shown, indicating a modulation between apoptosis and autophagy. Moreover, vitamin D_3, alone or in combination with ZK191784, administered before the oxidative stress, is able to prevent the loss of mitochondrial potential and the consequent cytochrome C release and caspase activation. In addition, vitamin D_3 alone or in combination with ZK191784 was able to prevent the MPTP opening caused by H_2O_2. These findings depend on changes of mitochondria-trapped calcein intensity and on effects of cyclosporin A, which inhibits MPTP opening, modulating the cyclophilin D activity [141].

Oxidative stress and the consequent mitochondrial fragmentation are involved in several neurodegenerative disorders as well [142–144]. Oxidative stress results from Fenton reaction, which generates ROS and consequently induces irreversible damage to DNA, RNA, proteins, and lipids [145]. Thus, there is compelling evidence that the neuroprotective action of vitamin D_3 is revealed through neuronal calcium regulation, antioxidative pathway, immunomodulation, and detoxification. Vitamin D_3 along with its metabolites influences directly or indirectly almost all metabolic processes such as proliferation, differentiation, apoptosis, inflammatory processes, and mutagenesis. Such multifactorial effects of vitamin D_3 can be a profitable source of new therapeutic solutions for two radically divergent diseases, cancer and neurodegeneration [146]. The role of vitamin D_3 to prevent iron damage in neuroblastoma cells BE(2)M17 has been recently studied [147]. In this research, the mechanisms involved in neurodegeneration, such as cell viability, ROS production, and the most common intracellular pathway have been studied. The BE(2)M17 cell line is an alternative neuronal cell model widely used in neuroscience research [148]. The beneficial effect of vitamin D_3 consists of a significant decrease in iron accumulation and ROS production, two important co-factors of neurodegeneration [149]. The effects of vitamin D_3 have been observed in BE(2)M17 cells through the activation of the VDR receptor and for this reason, the mechanisms underlying the protective effects of vitamin D_3 may be hypothesized to have genomic origin. Moreover, under these conditions, the survival pathway (ERKs) was also activated in presence of vitamin D_3. In addition, in these experiments Fe^{3+}-dependent activation of principal neurodegenerative biomarkers, such as p53 [150], Ki67 [151] and c-Myc [152], has been confirmed. A novel finding is represented by the demonstration that pre-treatment with vitamin D_3 is able to significantly counteract tumoral biomarker activation.

5. Conclusions

The results described herein highlight that vitamin D_3 stimulates endothelial cell proliferation and migration in a 3-D matrix and that these phenomena depend on NO production. Furthermore, this work adds new information to the debate on the benefits of vitamin D_3 supplementation. Indeed, it has been shown for the first time that vitamin D_3 may prevent endothelial cell death through the modulation of the interplay between apoptosis and autophagy. This effect is obtained by inhibiting superoxide anion generation, maintaining mitochondria function and cell viability, activating survival kinases, and inducing NO production. In the recent years, the knowledge about vitamin D_3 has improved and its implications have extended beyond its classical role in bone health in either fields of basic research as well as in human clinical trials, showing the relevance of the vitamin D_3 system. Until now, the available data are significant and confirm its essential role in several physiological and preventive functions. A greater understanding of vitamin D_3 system will shed new light on the use of vitamin D_3 supplementation in very promising fields such as tissue repair, wound healing, and prevention of the human angiogenic process. The former shows that the association between vitamin D_3 status and cardiometabolic outcomes is uncertain and that no clinically significant effect of vitamin D_3 supplementation at the dosages given is found. The latter suggests that vitamin D_3 dietary supplements at moderate to high doses may reduce cardiovascular disease risk. Vitamin D_3 along with its metabolites influences directly or indirectly almost all metabolic processes such as proliferation, differentiation, apoptosis, inflammatory processes, and mutagenesis. Such multifactorial effects of vitamin D_3 can be a profitable source of new therapeutic solutions for two radically divergent diseases as cancer and neurodegeneration [134]. The discussed results could be relevant in the light of the use of vitamin D_3 to promote supplementation or to adjust therapeutic strategies in neurodegenerative disorders.

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