Comparative Effectiveness of Vitamin D₃ and Dietary Vitamin E on Peroxidation of Lipids and Enzymes of the Hepatic Antioxidant System in Sprague – Dawley Rats

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Summary: The vitamin D – endocrine system has mostly been studied for its role in calcium and phosphorus metabolism and its possible role as an antioxidant has been neglected. This study attempts to elucidate the antioxidative properties of the prohormone with respect to vitamin E, a membrane antioxidant. Results herein show that D₃ treatment brought about similar reduction in the extent of lipid peroxidation and induction in superoxide dismutase (SOD) activity, as with vitamin E supplementation. While selenium dependent glutathione peroxidase (Se-dep. GPx) activity reflected no change with vitamin D₃ treatment, total GPx activity was more significantly influenced by vitamin D₃ than by vitamin E. The glutathione (GSH) content in the experimental rats also reflected similar changes. Vitamin E supplementation caused 8.57% increase in glutathione reductase (GR) activity, while vitamin D₃ decreased the concerned enzymes activity by 11.11%. Vitamin D₃ treatment also caused 25% increase in glucose 6-phosphate dehydrogenase (G6PD) activity. These data thus suggest that vitamin D₃ may function as an antioxidant in the liver in vivo and illustrate an effectiveness higher than that observed with vitamin E supplementation.

Introduction

Vitamin D₃ (cholecalciferol) is a prohormone that is synthesized in the skin of all higher animals by UV irradiation of 7-dehydro cholesterol. It is activated by hydroxylation reactions first in the liver and then in the kidney to give birth to its hormonal form, 1,25(OH)₂-cholecalciferol. The vitamin D-endocrine system has long been studied for its role in calcium and phosphorus metabolism [1, 2]. There is however, growing evidence on other nonclassical functions of the vitamin. This potent steroid hormone is known to regulate polyamine biosynthesis, lymphokine biosynthesis and biosynthesis of calcium binding proteins [3, 4, 5]. It is also well-known that vitamin D receptor is widely expressed not only in the classic target organs, but also in a variety of cell types such as hematopoietic cells and keratinocytes [1, 6]. Our understanding of the biological responses of the prohormone is far from being complete and there may be other important biological effects of vitamin D.

It is believed that presence of extensive systems of conjugated double bonds in a drug molecule is responsible for imparting antioxidant properties to the compound [7]. Besides, considerable reports are available on cholesterol as a membrane antioxidant [8] and many anticancer drugs like tamoxifen act as membrane antioxidants by structural mimicry of cholesterol [9, 10]. Although the ability of vitamin D₃ to
inhibit iron-dependent lipid peroxidation in liposomes has been discussed by Wiseman [11] and Wilson has studied the critical role of the vitamin in free-radical induced biological damage [12], the antioxidant properties of vitamin D₃ warrant further study.

On the other hand, considerable effort has been put into studying the antioxidant action of vitamin E. It has been established that vitamin E protects membrane and LDL against oxidative stress [13]. The cytoprotective mechanisms of vitamin E include quenching Reactive Oxygen Substances (ROS) and maintaining normal levels of thiols such as glutathione [14].

Our purpose in this study was to examine the relative ability of vitamin E and D₃ on hepatic antioxidant enzymes, glutathione (GSH) level and lipid peroxidation, measured as thiobarbituric acid-reactive substances (TBARS), in male Sprague-Dawley rats. Liver was chosen as the organ of study since it is known that many chemical changes are detectable in the liver before the onset of secondary physiological and nutritional changes anywhere else in the body [15]. Accordingly, hepatic antioxidant defence systems that directly or indirectly detoxify ROS, such as glutathione peroxidase (GPx, E.C. 1.11.1.9), glutathione reductase (GR, EC 1.6.4.2), superoxide dismutase (SOD, E.C. 1.15.1.1) and glucose 6-phosphate dehydrogenase (G6PD, E.C. 1.1.1.49) were estimated in the hepatic tissue of the experimental rats.

### Materials and Methods

**Animals**: Inbred, 5-week-old, male, Sprague-Dawley rats with a mean initial weight of 120 g were housed individually in wire-mesh cages in a room regulated for temperature (25±0.5°C), humidity (50–60%) and light (lights on 600–1800 h). Animals were cared for in accordance with guidelines established by the NIH guide for the Care and Use of Laboratory Animals. Food and water were always available. All rats were fed a semipurified basal diet as outlined in Table I. The rats were acclimatized to the laboratory conditions for a period of one week before start of experiment. They were divided into 4 groups, each consisting of 8 animals. Group 1, untreated control; group 2, supplemented with 1 g/kg of additional vitamin E (all-rac-α-tocopheryl acetate, Sigma) in the diet for 5 consecutive weeks; group 3, receiving 0.3 μg of vitamin D₃ (cholecalciferol, Sigma) dissolved in 100 μl of propylene glycol orally every Monday and Thursday for the same 5 weeks; group 4, respective vehicle control. The body weights and food consumption were determined at the same time interval for the 5 week duration of the experiment.

**Sample Collection**: Periodically 48 h after treatment with vitamin D₃, i.e., every Wednesday and Saturday, blood was collected by heart puncture from animals of all groups under ether anaesthesia and pooled for each group. Blood was allowed to clot at room temperature and centrifuged (4°C, 10,000 g, 5 min) to yield serum.

Following 5 weeks of treatment, the rats were deprived of food overnight and killed by decapitation. The livers were perfused with normal saline, rapidly removed and homogenized in four volumes of 0.25 M sucrose in a Teflon coated homogenizer. The homogenate was centrifuged at 2700 rpm (10,000 g) for 15 min in a Sorvall SS-44 rotor to pellet the nuclei and other tissue debris. The supernatant was taken for cytosolic assay.

**Serum Calcium level**: Sodium sulphate (0.05 g/ml) was added to the serum and the sample was digested with 2.3 mixture of concentrated H₂SO₄ and HNO₃ followed by heating gently to dryness. HCl was used to dissolve the residue and the serum Ca was finally recorded by Atomic Absorption Spectrophotometry.

**Serum 1α, 25 (OH): vitamin D level**: Serum levels of 1α, 25 (OH)₂D₃ were measured by the procedure of Mallon et al [16]. Serum for the analysis was pooled from several animals, when necessary. 1 ml of serum was extracted with methylene chloride: methanol and chromatographed on Sephadex LH-20 columns, previously washed with methanol. The 1α, 25 (OH)₂D₃ fraction was collected and measured by a competitive protein binding assay using the rachitic chick intestinal cytosol as the source of the binding protein.

**Enzymatic measurement**: GR was measured by the method of Carlberg and Mannervik [17] in the cytosolic fraction using oxidized glutathione (Sigma) as substrate and following the oxidation of NADPH spectrophotometrically at 340 nm. GPx was determined in an enzyme coupled reaction in the 10,000

### Table I: Composition of the semipurified diet (g/kg)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount (g/kg)</th>
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<tbody>
<tr>
<td>Wheat Flour</td>
<td>150</td>
</tr>
<tr>
<td>Gram flour</td>
<td>570</td>
</tr>
<tr>
<td>Groundnut flour</td>
<td>100</td>
</tr>
<tr>
<td>Skimmed Milk powder</td>
<td>50</td>
</tr>
<tr>
<td>Casein (minimum 80% protein)</td>
<td>40</td>
</tr>
<tr>
<td>Mineral mix</td>
<td>40</td>
</tr>
<tr>
<td>Vitamin mix**</td>
<td>2</td>
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</table>
g pellet and supernatant using H₂O₂ (Selenium dependent activity) and cumene hydroperoxide (Sigma) (total activity) as substrates by measuring the oxidation of NADPH at 340 nm [18]. Mn-SOD (10,000 g pellet) and Cu, Zn SOD (10,000 g supernatant) were determined spectrophotometrically according to the method of Marklund and Marklund [19]. Hepatic G6PD activity was assayed by adopting Lohr and Wallers' method [20]. Assay mixture contained glucose 6-phosphate (Sigma) and NADP (Sigma). Reading was taken at 365 nm and extinction increase at 2 min intervals were noted for 10 min. The rate of formation of NADPH was a measure of the enzyme activity.

**GSH measurement:** The GSH levels in the hepatic tissue were determined by the method of Anderson [21]. Assay mixture contained NADPH (Sigma) and 5, 5'-dithiobis (2-nitrobenzolic acid) along with the sample. Absorbance was measured at 412 nm. The amount of GSH was determined in μg/g tissue from a standard curve in which the GSH equivalents were present.

**Lipid peroxidation analysis:** As described by Ohkawa et al [22], a mixture of sodium dodecyl sulfate, acetic acid, thiobarbituric acid (TBA) and the sample at pH 3.5 was heated at 95°C for 60 min. After cooling, a mixture of n-butanol and pyridine were added and the solution was centrifuged (10,000 g, 15 min). The absorbance of the organic layer was measured at 532 nm. 1,1,3,3-tetramethoxypropanol (TMP) was used as an external standard since, TBA-colorimetric test is not specific for free malondialdehyde (MDA) and may express the total amount of many kinds of TBA-reactive substances (TBA-RS), including MDA and other aldehyde breakdown compounds in oxidized lipids [23, 24]. The level of lipid peroxide was expressed as nmol MDA/g wet weight of hepatic tissue. Figure 4 shows the relation between the amount of TMP and the absorbance at 532 nm to the relation between the volume of 10% homogenate of all the groups and the absorbance at 532 nm.

Protein was estimated by the method of Lowry et al [25].

**Statistical analysis:** Data were analyzed by least square ANOVA using STATPACK of the public domain. Differences between treatment means were considered significant at p < 0.05 [26] using the all-possible t-test matrix of the least square means generated. Data are presented as arithmetic mean ± SEM.

**Results**

For examining whether administration of 0.3 μg of vitamin D₃ bi-weekly for 5 consecutive weeks is having any toxic manifestation by induction of hypercalcaemia, serum Ca and 1, 25 (OH)₂ D levels were measured 48 h following each administration of D₃. As is evident from Figure 1, a sustained high normal level (20% on an average) of serum 1, 25 (OH)₂ D but a normal serum Ca level is brought about by D₃ supplementation at the studied dose. The vehicle control group and vitamin E supplemented animals showed no change in these parameters (not shown in Fig.).

The non-toxicity is further manifested in final body weights of the experimental rats. Treatment of D₃ caused a steady linear increase in body weight, the final weights being 8% higher in comparison to untreated controls. Vitamin E supplementation on the other hand slightly reduced (5%) the final body weights (Fig. 2). The body weight of the vehicle control group however, did not reflect any difference from the untreated controls (data not shown in Fig.).

Feed conversion efficiency (grams of body weight gained: gram of diet eaten) for the different groups was 0.32±0.01, control; 0.30±0.01, vitamin E; 0.34±0.01, vitamin D₃ and 0.31±0.01, vehicle. No group had feed conver-
Table II: Effect of supplementation of vitamin E and D3 on hepatic antioxidant enzymes in Sprague-Dawley rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GR$^1$ (μmol/mg min)</th>
<th>Total GPx$^1$ (μmol/mg min)</th>
<th>Se-Dep$^1$ (μmol/mg min)</th>
<th>SOY$^1$ (μmol/mg min)</th>
<th>GSTP$^*$ activity (mg Prot)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>70 ± 3$^a$</td>
<td>43 ± 4$^a$</td>
<td>30 ± 3$^a$</td>
<td>1112 ± 73$^a$</td>
<td>6.8 ± 0.4$^a$</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>76 ± 2$^b$</td>
<td>51 ± 2$^b$</td>
<td>36 ± 3$^b$</td>
<td>1337 ± 72$^b$</td>
<td>7.1 ± 0.5$^b$</td>
</tr>
<tr>
<td>Vitamin D3</td>
<td>63 ± 3$^c$</td>
<td>57 ± 2$^c$</td>
<td>29 ± 2$^c$</td>
<td>1395 ± 78$^c$</td>
<td>8.5 ± 0.8$^c$</td>
</tr>
<tr>
<td>Vehicle</td>
<td>71 ± 2$^d$</td>
<td>42 ± 3$^d$</td>
<td>31 ± 4$^d$</td>
<td>1101 ± 79$^d$</td>
<td>7.0 ± 0.3$^d$</td>
</tr>
</tbody>
</table>

$^1$ Values are mean ± S.E., n = 8. Values within the same column not sharing a common superscript are significantly different (p < 0.05)

$^2$ Cytosolic glutathione reductase (EC 1.6.4.2) activity per milligram of protein

$^3$ Total glutathione peroxidase (EC 1.11.1.9) activity per milligram of protein, measured using cumene hydroperoxide as substrate

$^4$ Selenium dependent glutathione peroxidase (EC 1.11.1.9) activity per milligram of protein, measured using H$_2$O$_2$ as substrate

$^5$ Manganese superoxide dismutase (EC 1.15.1.1) activity per milligram of protein

$^6$ Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) activity per milligram of protein

sion efficiency significantly different from the control group (not shown in Fig.).

Data in Table II show that while vitamin E supplementation caused a 8.57% increase in GR activity in comparison to the untreated controls, vitamin D3 treatment resulted in 11.11% decrease in GR activity. Rats receiving vitamin D3 also caused a 32.5% increase in total (cumene hydroperoxide) GPx, although no change was reflected on the selenium dependent (H$_2$O$_2$) GPx specific activity. While vitamin E supplementation resulted in a 20% increase in the Se-dependent GPx activity, the total GPx activity was much less influenced showing an increase of 18.6% only. SOD activity increased by 20.2% and 25.4% by vitamin E and vitamin D3 treatment respectively. G6PD activity however did not reflect any significant changes with vitamin E supplementation whereas, D3 treatment brought about a 25% increase in G6PD activity.

The hepatic GSH content in both vitamin E and D3 treated rats, shown in Figure 3, exhibited an increase, although in the latter this increase was more pronounced (49.5%).

As is evident from Figure 5, the extent of lipid peroxidation in the hepatic tissue was significantly and similarly decreased by both vitamin E and D3.

Discussion

In the last two decades it has become abundantly clear that the reactions forming ROS can and do occur inside cells. Thus, aerobic organisms as a whole are potentially at risk and their survival will depend on ameliorating or eliminating ROS [27]. It therefore seems reasonable to assume that most cells are equipped with regulatory mechanisms that can sense a high oxidative stress potential and consequently induce higher levels of defence activities [28]. Because free radical reactions are essential for maintaining life, the key issue is the maintenance of an appropriate balance between peroxidative events that are necessary and those that are excessive [29]. This in part explains the role of antioxidant enzymes. SOD catalyzes the conversion of superoxide radicals to H$_2$O$_2$ [30] which is then destroyed by a coupled enzyme system, GPx and GR [31]. The latter two en-

Figure 3: Hepatic GSH content in the vitamin E and D3 treated rats against their control counterparts. Values are mean ± S.E., n = 8. Significant difference at p < 0.05 is represented by *.
Figure 4: Relationship between the amount of 10% tissue homogenate and absorbance at 532 nm in reference to TMP as an external standard. Values of the four different groups (1–4) are expressed as mean ± S.E., n = 8.

Figure 5: Extent of lipid peroxidation in rats receiving vitamin D₃ and vitamin E with respect to the control animals. The level of lipid peroxides is expressed in terms of nmol MDA/g wet weight, which was calculated from the absorbance at 532 nm using TMP as an external standard, and expressed mean ± S.E., n = 8. Significant difference at p < 0.05 is represented by *

Enzymes reduce H₂O₂ to water at the expense of NADPH, using GSH as an electron donor. The enzymes of the hexose monophosphate shunt regenerate the NADPH [32]. A further defence against oxidative damage is the presence of antioxidant in the cell membrane. The best studied of these is vitamin E [33].

Cholecalciferol was found to be an effective antioxidant in this study and was interestingly more potent than vitamin E in boosting the indices of the antioxidant defence. The content of GSH which represents the overwhelming part of intra as well as extracellular soluble thiols [34], was elevated by 49.5% in D₃ treated rats. Since, thiol homeostasis is required to guarantee basic functions and defence mechanisms against xenobiotics [35], this increase in hepatic GSH content indicates a protective role of the vitamin in fighting free radical damage. The increase however possibly does not result in direct metabolic changes since, the GSH dependent enzymes operate under optimal conditions with regard to GSH [34]. Vitamin E supplementation caused an insignificant increase of GSH content.

The data on the enzymes related to free radical detoxification similarly suggest that vitamin D₃ may function as an antioxidant in liver in vivo and illustrate an effectiveness higher than that observed with vitamin E supplementation. The elevation in the activity of GR, the enzyme that transfers electrons to oxidized glutathione, is indicative of oxidative stress [36]. D₃ treatment significantly lowers GR activity, thereby maintaining the system in a low-stress condition. Induction in total GPx and SOD activity also strengthens this possibility. The 25% increase in G6PD activity warrants a ready abundance of reducing equivalents in times of need. On the other hand, MDA formation was limited to a similar extent by vitamin D₃ and vitamin E treatment. D₃ is likely to act as a membrane antioxidant by stabilizing the membrane against lipid peroxidation via an interaction between its hydrophobic rings and the saturated, monounsaturated and polyunsaturated residues of the phospholipid fatty acid side chains, which decreases membrane fluidity [11], as has been proposed for cholesterol [37].

Wiseman has shown the ability of vitamin D in inhibiting iron dependent lipid peroxidation in liposomes and has suggested that this may be of importance in protecting the membranes of normal cells against free radical induced oxidative damage, as it is possible that this highly lipophilic compound may accumulate in membranes to achieve the concentrations found to inhibit lipid peroxidation [11]. Herein, we have additionally established the effectiveness of vitamin D₃ in modifying GSH and GSH-dependent enzymes.

Thus, results herein show that vitamin D₃ treatment causes elevated levels of GPx, SOD and a significant decrease in lipid peroxidation.
Interestingly, these enzymes have been shown to diminish in all tumors studied to date [38, 39] and enhancement of lipid peroxidation has been suspected of contributing to the process of cancer development [40]. Vitamin D₃ could thus pose as an effective antineoplastic agent in the near future. Several epidemiological studies have indeed established the protective role of vitamin D₃ against colon cancer [41, 42] and this finding has been strengthened by in vitro and in vivo studies [43, 44]. However, a prompt verification of the antioxidant potential of D₃ in a defined chemical carcinogenesis model warrants further study.

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References


