Vitamin D reduces deposition of advanced glycation end-products in the aortic wall and systemic oxidative stress in diabetic rats

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Aims: Vitamin D may have an important role in reducing the risk of cardiovascular disease. Advanced glycation end-products (AGEs) such as Nε-(carboxymethyl)lysine (CML), have been implicated in diabetic vascular complications via oxidative stress-mediated pathways. We investigated the potential protective effect of vitamin D on CML accumulation in the diabetic aortic wall. To test the effects of vitamin D on systemic oxidative stress we also assessed liver oxidative stress index (OSI) and serum total antioxidant capacity (TAC).

Methods: Male Wistar rats were assigned to three groups: control, untreated diabetes, and diabetes + cholecalciferol. Diabetes was induced by streptozotocin, followed by oral administration of cholecalciferol (500 IU/kg) for 10 weeks in the treatment group. Aortic CML accumulation was determined by ELISA and immunohistochemical assays. OSI was assessed by measuring TAC and the level of total peroxides in the liver and serum using colorimetric assays.

Results: Untreated diabetes was associated with significantly elevated CML levels in the aortic wall (19.5 ± 3.3 vs 10.2 ± 4.7 ng/mL), increased liver OSI (6.8 ± 1.9 vs 3.1 ± 0.7), and reduced serum TAC (0.4 ± 0.1 vs 0.8 ± 0.3 mmol Trolox/L), in comparison with the control group. Cholecalciferol significantly blocked the accumulation of CML in the aortic wall (10.4 ± 8.4 vs 19.5 ± 3.3 ng/mL), decreased liver OSI (4.2 ± 1.4 vs 6.8 ± 1.9), and improved serum TAC (1.0 ± 0.2 vs 0.4 ± 0.1 mmol Trolox/L), compared with the untreated diabetic group.

Conclusions: Streptozotocin-diabetes resulted in increased deposition of AGEs and increased oxidative stress in the serum and liver. Vitamin D supplementation may provide significant protection against oxidative stress-mediated vascular complications in diabetes.

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1. Introduction

Advanced glycation end-products (AGEs) are irreversibly formed during glycation and oxidation of proteins [1]. Because of its abundance in uncontrolled diabetes mellitus (DM), glucose is assumed to be a major source of glycation and AGE formation. AGEs can form covalent cross-links between the extracellular matrix proteins in the vascular wall and alter their function, particularly of collagen and elastin [2,3]. AGE cross-linking of collagen has been associated with increased stiffness of the arterial wall and impaired arterial haemodynamics [4]. Increases in the levels of Nε-(carboxymethyl)lysine (CML), a major antigenic structure of AGEs [5], have been correlated with the severity of diabetic complications [6,7], suggesting that CML may be an important biomarker in the assessment of vascular integrity in DM. Considering the emerging evidence about the adverse effects of AGEs on the vascular properties, therapies aimed at inhibiting AGEs may provide significant improvement of diabetic vascular complications.

Oxidative stress is regarded as an important factor in the pathogenesis and progression of DM and its associated cardiovascular complications [8]. In diabetes, persistent hyperglycemia is associated with increased production of reactive oxygen species (ROS) [9] with concomitant depletion of intrinsic antioxidant defense mechanisms [10], rendering the organism more susceptible to oxidative damage. The imbalance between oxidants and antioxidants could be largely responsible for the functional and morphological damages to the blood vessels associated with uncontrolled hyperglycemia [11]. Oxidative reactions also enhance the formation of AGEs and their accumulation in cardiovascular tissue [12], raising a potential link between diabetic vascular complications and deposition of AGEs.

Agents that enhance the antioxidant defense and reduce the damage caused by oxidative stress to the blood vessels may be beneficial in the treatment of diabetic vascular complications. Several studies have demonstrated that vitamin D may reduce lipid peroxidation [13,14] and maintain a steady level of glutathione (GSH), a potent intracellular antioxidant [15]. Calcitriol, the active form of vitamin D, has been shown to improve vascular endothelial function by modulating the expression of radical generating and scavenging enzymes, thus preventing the overproduction of ROS [16]. In our previous study, we demonstrated that chronic supplementation of vitamin D improves aortic wall remodeling in streptozotocin-induced diabetes [17]. To test the possible ways how vitamin D may have protective effects concerning diabetes-induced vascular abnormalities we investigated in the current study the effect of vitamin D on CML deposition in the aortic wall, and its potential antioxidative influence by assessing systemic oxidative stress-related parameters in the serum and liver in a rat model of type 1 DM.

2. Methods

2.1. Animals

Male Wistar rats (n = 24, age 4 months), purchased from Harlan Laboratories (Harlan Laboratories, Inc., The Netherlands), were used in the experiments. The animals were housed in a room maintained at 21 ± 2 °C under a standard 12-h light/12-h dark cycle and were given normal rat chow and tap water ad libitum. All experimental procedures were approved by the Estonian National Board of Animal Experiments and were conducted in accordance with the European Communities Directive (86/609/EEC).

2.2. Experimental protocol

Rats were randomly assigned to one of three groups: control, diabetic, and cholecalciferol-treated diabetic group. Diabetes was induced by an intraperitoneal administration of streptozotocin (STZ) 50 mg/kg (Sigma-Aldrich, St. Louis, MO, USA), freshly dissolved in 0.9% saline solution. Blood samples from the tail vein were obtained 48 h later and glucose levels were measured with a glucometer (Glucocard X-meter, Arkray Inc., Japan). All rats in the diabetic groups had blood glucose level >15 mmol/L. Supplementation of cholecalciferol (Sigma-Aldrich, St. Louis, MO, USA) 12.5 μg (500 IU) kg⁻¹ body weight, dissolved in 0.3 ml olive oil, was started immediately after confirmation of diabetes. Cholecalciferol was administered orally every other day for 10 weeks.

2.3. Laboratory parameters

At 10 weeks, the animals were anaesthetised with a mixture of fentanyl (0.07 mg/kg, Gedeon-Richter Plc., Hungary), midazolam (5 mg/kg, Roche Pharma AG, Germany), and ketamine (75 mg/kg, Vetoquinol Biowet Sp. z.o.o., Poland) administered subcutaneously. Blood samples were taken from the tail vein for assessment of glucose levels. Anaesthetised animals were subjected to cardiac puncture for blood withdrawal, followed by cervical dislocation. One part of blood sample was used for assessment of glycated haemoglobin (HbA1c) and glucose levels, and the remaining portion was centrifuged at 3000 rpm for 15 min to obtain serum. Samples of liver and aortic tissue were collected, soaked in ice-cold 0.9% NaCl, snap-frozen in liquid nitrogen, and stored at −60 °C until use.

Calcium (Ca) concentration in the serum was determined by a colorimetric test (Calcium liquidor, HUMAN Gesellschaft für Biochemica und Diagnostica mbH, Germany). Serum albumin levels were measured using a colorimetric test (Albumin liquidor, HUMAN Gesellschaft für Biochemica und Diagnostica mbH, Germany). Serum Ca levels were corrected for albumin concentrations using the following formula: corrected Ca = serum Ca + 0.02 × (40 − serum albumin) [18].

Serum 25-hydroxyvitamin D (25(OH)D) levels were measured using a radioimmune assay (25-Hydroxyvitamin D, 125I Ria Kit, Diasorin Corporation, USA).

2.4. Oxidative stress markers

Liver samples were homogenised in ice-cold 0.9% NaCl solution and centrifuged at 15,000 × g for 10 min at 4 °C. The insoluble pellets were discarded and the supernatants were used for analysis. Total peroxide concentrations (TPX) were measured with an OxyStat colorimetric assay kit (Biomedica Gruppe, Austria), which assesses the colour
change produced by the reaction of total peroxides in the sample with peroxidase. The data were expressed as nmol/mg protein, with protein concentration measured spectrophotometrically using the Lowry method [19].

Total antioxidant capacity (TAC) was measured in the serum and liver samples using a colorimetric assay (Randox Laboratories Ltd., UK). This assay is based on the decolorisation of 2,2'-azinobis(3-ethylbenzothiazoline 6-sulfonate) (ABTS) radical action. The TAC value was expressed as an equivalent of the millimolar concentration of Trolox (a soluble vitamin E analogue) solution. Oxidative stress index (OSI), an indicator of the redox balance between oxidation and antioxidation, was expressed as the percent ratio of TPX to TAC [20].

Aortic strips (10 mm) were homogenised in ice-cold 0.9% NaCl solution, using a blade type homogeniser (Tekmar Tissumizer, Cincinnati, OH, USA) and centrifuged at 10,000 g for 10 min at 4 °C. The insoluble pellets were discarded and the supernatants were used for analysis. The concentration of CML was assessed using a commercial enzyme-linked immunosorbent (ELISA) test OxiSelect™ N-epi-l-(Carboxymethyl) Lysine ELISA Kit (Cell Biolabs, Inc., San Diego, CA, USA). Ten μg/ml of total protein extracts were adsorbed onto a 96-well plate at 4 °C overnight. The absorbance of each well was read using a microplate reader (Tecan Sunrise, Tecan GmbH, Austria) using 450 nm as the primary wavelength. The concentration of CML was calculated by comparison to a standard curve consisting of known concentrations of CML-BSA. Results are presented as nanogram of CML per millilitre of solution.

2.5 Immunohistochemistry

Three-μm thick paraffin sections mounted on poly-L-lysine coated SuperFrost slides (Menzel-Gläser, Germany) were deparaffinised and rehydrated. Peroxidase activity was blocked by 0.6% H₂O₂ (Merck, Germany) in methanol (Merck, Germany). Then the sections were washed in tap water and in PBS (pH = 7.4; Gibco, Invitrogen, USA) for 10 min, treated with normal 1.5% goat serum (Gibco, Invitrogen Corporation, USA) for 20 min at room temperature and incubated with the first antibody: anti-carboxymethyl lysine (mouse monoclonal antibody [CML26], abcam, UK) diluted 1:50 overnight at 4 °C in the humidity chamber. On the next day the sections were incubated with the biotinylated mouse anti-mouse secondary antibody for 30 min at room temperature. After a wash step the sections were incubated with the avidin-biotin peroxidase complex ELITE system (Vectastain Elite ABC Kit, Vector Laboratories Inc., Burlingame, USA) for 30 min. Peroxidatic activity was detected with 3,3′-diaminobenzidine [DAB] (Vector Laboratories Inc., USA) and the sections were counterstained with hemalaun, dehydrated and mounted with DPX (Fluka, Switzerland). The labelling was expressed on a semi-quantitative scale ranging from 0 to 4 (0 — no staining, 1 — weak staining, 2 — moderate staining, 3 — strong staining, 4 — very strong staining). Two independent observers in a blinded fashion performed the evaluation. Immunohistochemistry negative controls were performed by omitting the primary antibody (mouse IgG was used in place of the primary antibody).

2.6. Statistical analysis

Results are expressed as means ± standard deviation (SD). Differences between the groups were evaluated using the one-way analysis of variance (ANOVA) followed by Tukey’s post hoc analysis for multiple comparisons of group means. Semi-quantitative data were compared by the Kruskal–Wallis one-way ANOVA followed by Mann–Whitney U test. Differences were considered to be statistically significant when p was <0.05. All statistical comparisons were performed with the Statistica software (version 8, StatSoft, USA).

3. Results

The basic parameters and biochemical results are presented in Table 1. Rats in both diabetic groups presented with significantly lower body weights and increased levels of blood glucose and HbA1c. Serum 25(OH)D levels were significantly decreased in untreated diabetic rats and completely restored by vitamin D supplementation. Serum Ca and albumin-corrected Ca levels were not different between all groups, indicating that calcium homeostasis was not affected by short-term changes in circulating vitamin D levels.

Untreated diabetes produced significantly higher levels of CML in the aortic wall homogenates and OSI in the liver (Figs. 1 and 2). The levels of serum TAC were significantly decreased in untreated diabetic rats compared to non-diabetic rats (Fig. 3).

| Table 1 – Body weights and serum laboratory values at the end of the study. |
|-----------------|-----------------|-----------------|--------------------|-----------------|-----------------|-----------------|-----------------|
| Group           | Body weight (g) | Blood glucose (mmol/L) | HbA1c (%)       | 25(OH)D (nmol/L) | Calcium (mmol/L) | Albumin (mg/L) | Corrected calcium (mmol/L) |
| Control         | 456 ± 26        | 6.3 ± 1.6         | 4.0 ± 0.1       | 140 ± 21        | 2.6 ± 0.2       | 39.3 ± 2.7     | 2.6 ± 0.3        |
| Diabetes        | 374 ± 54        | 28.3 ± 3.9        | 10.3 ± 0.7      | 108 ± 38        | 2.5 ± 0.2       | 30.8 ± 3.2     | 2.6 ± 0.3        |
| Diabetes + vitamin D | 348 ± 43        | 28.5 ± 5.9        | 9.5 ± 1.3       | 494 ± 125       | 2.6 ± 0.3       | 33.4 ± 5.1     | 2.7 ± 0.2        |

Data are expressed as mean ± SD (n = 8 per group).
HbA1c, glycated haemoglobin; 25(OH)D, 25-hydroxyvitamin D; Corrected calcium, calcium concentrations adjusted to albumin concentrations using the following formula: corrected calcium = serum calcium + 0.02 × (40 – serum albumin).

* p < 0.05 vs control.
* p < 0.01 vs control.
* p < 0.001 vs diabetes + vitamin D.
Vitamin D effectively prevented CML accumulation in the aortic tissue (Fig. 1) and reduced the levels of OSI in the liver, compared to untreated diabetic rats (Fig. 2). Furthermore, significant improvement of serum TAC was observed in the diabetic treated group where TAC was restored to a higher level than that in the control group (Fig. 3).

Immunohistochemical detection of CML demonstrated strong immunostaining in all layers of the aortic wall in the untreated diabetes group (Fig. 4). In the diabetic treated group, strong staining was still seen in the intima and adventitia, but the thickest layer, the media, showed moderate staining. Similar staining pattern was found in the control group, where immunostaining was moderate in intima and adventitia, while the medial layer stained weakly (Table 2).

4. Discussion

In our previous study, we demonstrated that chronic supplementation of vitamin D to STZ-diabetic rats provides significant protection against diabetes-induced alterations in the arterial structure [17]. In this study, we have assessed the effects of vitamin D on the aortic wall deposition of AGEs and oxidative stress status in the blood and liver in a rat model of type 1 DM. The major findings of this study extend our previous data regarding that the vasoprotective effects of vitamin D could be mediated, at least in part, via inhibition of the accumulation of AGEs in the aortic wall in the context of improved systemic antioxidant capacity and reduced oxidative stress in the liver, the key organ in the metabolism and circulation of vitamin D.

AGE formation in DM could be responsible for development of vascular complications by several mechanisms. In the arterial wall, accumulation of AGEs may adversely affect vasodilatation either by increased stiffness of structural proteins [3,4] or by disturbing the smooth muscle response to nitric oxide [21]. The levels of AGEs in tissues reflect the severity of these complications while therapeutic interventions aimed at reducing AGEs may inhibit or delay their progression [22,23]. Our findings of increased deposition of CML, the major advanced glycation adduct, in the aortic wall induced by untreated experimental DM are consistent with

**Fig. 1** – Diabetes was associated with significantly higher levels of CML in the aortic wall that was prevented by vitamin D supplementation. CML, N'-(carboxymethyl)lysine. Data are mean ± SD (n = 8 per group).

**Fig. 2** – Oxidative stress index in the liver was significantly increased in untreated diabetes and improved by vitamin D. Oxidative stress index is expressed as the percent ratio of TPX to TAC. TPX, total peroxide level; TAC, total antioxidant capacity. Data are mean ± SD (n = 8 per group). \( PS = \text{non-significant difference.} \)

**Fig. 3** – Serum total antioxidant capacity was significantly decreased in diabetic rats and completely restored by treatment with vitamin D. Data are mean ± SD (n = 8 per group).

**Table 2** – Estimation of the immunostaining intensity of carboxymethyl lysine in the layers of the aortic wall.

<table>
<thead>
<tr>
<th>Group</th>
<th>Intima</th>
<th>Media</th>
<th>Adventitia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.40 ± 0.41</td>
<td>0.79 ± 0.37</td>
<td>1.96 ± 0.39</td>
</tr>
<tr>
<td>Diabetes</td>
<td>2.79 ± 0.39</td>
<td>2.07 ± 0.45</td>
<td>2.86 ± 0.24</td>
</tr>
<tr>
<td>Diabetes + vitamin D</td>
<td>2.79 ± 0.39</td>
<td>1.30 ± 0.27</td>
<td>2.50 ± 0.35</td>
</tr>
</tbody>
</table>

The staining intensity was expressed by a scale ranging from 0 to 4 (0 – no staining, 1 – weak staining, 2 – moderate staining, 3 – strong staining, 4 – very strong staining). The scores were summed and the values are presented as means ± SD (n = 8 per group).

\( p < 0.01 \) vs control.

\( p < 0.01 \) vs untreated diabetes, Mann–Whitney U-test.
reports from animal [3,24] and clinical studies [4,23] describing increased levels of AGEs in the arterial wall associated with structural and functional disturbances. We confirmed these results by using a second, immunohistochemical method, and found that CML was localised in the intima, media, and adventitia with prominent stainings in the intimal and adventitial layers.

It is an open question whether the accumulation of AGEs in vascular tissue can be attributed mainly to the chronically increased blood glucose levels and to what extent is elevated oxidative stress involved in this process. It has been proposed that persistent hyperglycaemia promotes a pro-oxidant environment via auto-oxidation of glucose [25] which exhausts the potential of the endogenous antioxidant system [9,10]. AGEs also have a causative role in oxidative damage through binding to the receptor for AGEs (RAGE). The binding of AGEs to RAGE initiates many of the downstream effects, including superoxide radical generation and apoptosis [26,27]. In agreement with previous studies [9,28,29], we show that the susceptibility to oxidation is significantly increased in the diabetic liver, as evidenced by increased levels of OSI. Moreover, significantly lower levels of serum TAC in the untreated diabetic rats reflect a severely compromised systemic antioxidant response to the increased oxidative stress. TAC assay is used widely to assess the synergistic effects of different antioxidants to neutralise free radicals [30]. Our results are in accordance with those reported by other investigators, demonstrating decreased plasma radical-trapping potential in experimental [28] and clinical diabetes [10,31].

Certain AGEs (e.g. pentosidine) are directly derived from the non-enzymatic reactions between proteins and carbohydrates, while a combination of glycation and oxidation reactions is required for the formation of CML [32]. In our study, vitamin D inhibited the CML deposition in the medial layer of the aortic wall in the presence of ongoing hyperglycaemia. This implies that vitamin D is involved in other important mechanisms of CML formation. To our best of knowledge, this is the first study demonstrating such an inhibitory effect of vitamin D on CML accumulation. The mechanism of this effect remains to be established, but it is possible that vitamin D interferes with the formation of reactive oxygen species by upregulation of antioxidant enzymes [13,33]. Furthermore, in vitro studies have shown that vitamin D reduces endoplasmic reticulum stress [34] and downregulates the expression of RAGE [35].
The liver is central to the vitamin D metabolism and circulation in that liver converts absorbed vitamin D into 25(OH)D, the circulating form vitamin D, which is the best indicator of vitamin D status in the organism [36]. Thus, we aimed to assess the impact of vitamin D pooled in the liver on the increased oxidative stress, as seen in the diabetic rats. We found that supplementation of vitamin D was able to reduce OSI to the level that of normal rats, clearly demonstrating the antioxidative potential of vitamin D. These results are in line with those by Hamden et al. [13] who showed that oxidative stress in the diabetic liver may be improved by calcitriol, the active form of vitamin D. Moreover, we found that treatment of diabetic rats with vitamin D restored the serum TAC to a level that was significantly higher than that found in control rats. Detailed explanation to the antioxidative effects of vitamin D cannot be provided, but these may include stabilisation of the plasma membrane against lipid peroxidation [12] or upregulation of antioxidant systems, including GSH, GSH peroxidase, and superoxide dismutase, via its nuclear receptors [13,33].

Our findings underscore the importance of oxidative and antioxidative status in the development of diabetic vascular complications. Indeed, among diabetic populations, a considerable variance in the rates of AGE accumulation, despite similar blood glucose and HbA1c levels, has been demonstrated which may be attributed to individual variations in oxidative stress status [6]. Furthermore, antioxidants are known to decrease CML formation [37] which, again, emphasises the essential role of oxidative stress in the advanced glycation processes.

In conclusion, the findings of the present study indicate that vitamin D may have a role in reducing different diabetes-related complications. Particularly, we demonstrate that treatment with vitamin D reduces the accumulation of AGEs in the medial layer of the aortic wall and oxidative stress at a systemic and end organ level. Thus, our results support the understanding that vitamin D supplementation provides an important protection from the oxidative damage associated with the development of diabetic vascular complications.

Conflict of interest

The authors declare that they have no conflict of interest.

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