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Research Article

EFFICACY OF COSUPPLEMENTATION THERAPY WITH VITAMINS B_9, B_{12}, AND D ON ENDOTHELIAL DYSFUNCTION IN STREPTOZOTOCIN-INDUCED DIABETIC RATS

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ABSTRACT

Objective: This study evaluated the effects of Vitamins D, B_9, and B_{12} given individually or combined in ameliorating some biochemical parameters related to endothelial dysfunction in diabetic rats.

Methods: A total of 50 Sprague-Dawley male rats were divided into five groups: Control, diabetic, diabetic received Vitamin D, diabetic received Vitamins B_9 and B_{12}, and diabetic received Vitamins B_9, B_{12}, and D. At the end of 6 weeks, the rats were sacrificed and a set of assays was carried out to determine: Fasting blood sugar (FBS), lipid profile, nitric oxide (NO), homocysteine (Hcy), malondialdehyde (MDA), and serum levels of Vitamins B_9, B_{12}, and D.

Results: Diabetic rat received Vitamin D and diabetic rat received Vitamins B_9 and B_{12} had a significant decline in the levels of FBS, lipid profile, and Hcy with reduced MDA ($p<0.05$) release but significant increase in NO level. On the same hand, diabetic rat received combined supplementation of Vitamins B_9, B_{12}, and D had more pronounced effect ($p<0.00$).

Conclusion: Given these findings, the combined vitamins therapy had antiatherosclerotic effects by inhibiting lipid peroxidation and stimulating NO production, resulting in amelioration the endothelial dysfunction in diabetic rat.

Keywords: Endothelial dysfunction, Diabetes, Vitamin D, Vitamin B9, Vitamin B_{12}.

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INTRODUCTION

The endothelium plays a pivotal role in multiple complex physiological mechanisms that regulate vascular tone, maintain blood fluidity, control coagulation, and platelet aggregation. On the other hand, it limits proliferation of smooth muscle cell and inflammation [1]. It is well established that diabetes is considered as a vascular disease as it affects macro- and microcirculation of many vascular beds. Hyperglycemia causes vascular damage through several mechanisms: Enhancing the formation of advanced glycation end products (AGEs), protein cross-linking, and ROS formation [2]. AGEs decrease nitric oxide (NO) bioavailability, and eNOS expression increases intracellular enzymatic superoxide production and also increases expression of ET-1. Subsequently, this causes an imbalance between NO and ET-1 to favor vasoconstriction and endothelial dysfunction [3]. Studies have suggested that Vitamin D deficiency is associated with diabetes [4]. Nevertheless, little is known about the precise mechanism(s) of Vitamin D on the endothelial system. Vitamin D has a potential protective effect on the vascular endothelium [5]. Endothelial cells express Vitamin D receptors and 1α-hydroxylase activity, thus allowing autocrine production of 1, 25-dihydroxyvitamin D (1, 25(OH) D2), the biologically active form of Vitamin D [6]. Apart from the capacity to modulate the effects of pro-inflammatory cytokines on the vascular endothelium, decrease the expression of endothelial adhesion molecules, Vitamin D may also exert antioxidant properties. Vitamin D may be involved in repairing damaged certain bone marrow-derived cells, the endothelial progenitor cells that have an important role in endothelialization and vascular repair following injury [7]. Micronutrients that have an antioxidant function (e.g., Vitamins B_9 and B_{12}) are very important in the development of the disease and its complications; they have an important role in regulating the metabolic process of homocysteine (Hcy), which generates potent ROS free radical that inhibits glutathione peroxidase causing vascular oxidative stress responsible for reduced NO bioavailability [8]. Previous studies have suggested that Vitamins B9 and B12 are associated with the development of adverse serum lipid profiles and stroke in patients with hypertension and diabetes [9]. Deficiencies in both vitamins cause elevated serum Hcy by inhibiting its conversion to methionine [10]. Persistent elevated serum Hcy has been also shown to be a risk factor for hypertension [11], diabetes, and related complications [12]. The aim of this study was to examine the effect Vitamins D, B9, and B12 given individually or combined in ameliorating some biochemical parameters related to endothelial dysfunction in streptozotocin (STZ)-induced diabetic rats.

METHODS

Chemicals

STZ, Vitamin D (1, 25-dihydroxyvitamin D3), Vitamins B9 (folic acid), and B12 purchased from Sigma Chemical Co. (St. Louis, Mo, U.S.A). All vitamins and drug were dissolved in sterile normal saline 20 min before use.

Experimental protocol

All animals received human care in compliance with guidelines of the Ethical Committee of National Research Centre, Giza, Egypt. 50 SD male rats with an initial weight 200–210 g and 6 months of age kept in standard conditions of temperature and light; they were given free access to a standard diet and water *ad libitum*. They were randomly divided into five groups of 10 animals each and treated for 6 weeks as follows: Control group was injected with sodium citrate buffer (50 mg/kg, subcutaneous), diabetic group was injected with a single subcutaneous dose of STZ (50 mg/kg), the animals considered diabetic if fasting glucose level was ≥200 mg/dl after 48 h of the injection [13], diabetic group received Vitamin D (6 ng/kg/day) [14], diabetic group

received both of Vitamins B9 (50 mg/kg/day using intragastric tube) [15] and B12 (15 µg/kg intraperitoneal twice a week) [16], and the fifth, diabetic group received Vitamins D, B9, and B12 as previously described.

Biochemical analysis

At the end of the experiment, animals kept fasting for 12 hrs, then anesthetized under light ether anesthesia. Blood samples collected from dorsal aorta, fasting plasma glucose assessed immediately by the glucose oxidase method (BioMerieux, Marcy l'Etoile, France) [17]. Triglycerides, total cholesterol, high-density lipoprotein-cholesterol, and low-density lipoprotein-cholesterol measured by the colorimetric enzymatic assays using kits supplied from Biocon, Diagnostic (Germany) [18-20]. Serum levels of Vitamins D, B9, and B12 measured by ELISA technique using kit provided from RayBiotech, Inc., USA.

Determination of serum Hcy

Hcy estimated by high-performance liquid chromatography (HPLC) system, Agilent technologies 1100 series equipped with a quaternary pump (G131A model).

Sample extraction

Serum samples (400 µl each) treated with 30 µl of 1.2 mol/L trichloroacetic acid mixed well and incubated in ice bath for 30 mins to precipitate protein. After centrifugation for 20 mins at 4000 rpm and 4°C, supernatants filtered through hydrophilic 0.45 µm polyvinylidene fluoride membrane filter.

HPLC operation condition

Filtered supernatants (50 µl each) injected into HPLC; separation achieved on reversed phase column (C18; length 25 cm, diameter 0.46 cm, and I.D. 5 µm). The mobile phase consisted of 40 mmol/L sodium phosphate monobasic monohydrate, 8 mmol/L heptanesulfonic acid, and 18% (v/v) methanol. The pH of the mobile phase adjusted to 3.1 by addition of phosphoric acid then filtered 2 times through a 0.45 µm membrane filter. The mobile phase was then delivered at a flow rate of 1 ml/min at 40°C. UV detection performed at 260 nm. Serial dilutions of standards injected into HPLC, and their peak areas determined. A linear standard curve constructed by plotting peak areas versus the corresponding concentrations. Concentrations in samples were successfully derived from the standard curve [21].

Preparation of tissue

Aorta tissue samples (100 mg/ml buffer) homogenized in 50 mM phosphate buffer (pH 7.0) and then centrifuged at 10,000 rpm for 15 mins; the supernatant used for measurement of NO and malondialdehyde (MDA). Tissue nitrite/nitrate (NO) measured using ELISA microplate reader and employing the modified Griess method [22]. Lipid peroxidation products in the aorta homogenates assayed by measuring the level of MDA [23].

Statistical analysis

Data analysis was carried out using the Statistical Package for the Social Sciences (SPSS) program, version 16 and Microsoft Excel 2007. Data presented as means ± standard error (SE). One-way ANOVA and Student's t-test estimated, $p<0.05$ was regarded as statistically significant.

RESULTS

Effect of vitamin supplements on serum blood sugar and lipid profile

The mean fasting blood sugar (FBS) levels of groups received Vitamin D individually or in combination were significantly low as compared to the diabetic group, but the reduction was more pronounced in the diabetic group that received Vitamins D, B9 and B12 as compared to the other groups. Mean serum lipid profile levels were significantly high in the diabetic group. Regarding groups received Vitamin D individually or in combination the mean serum lipid profile levels were low, but the reduction was more pronounced in the diabetic group received the combination of the three vitamins, Table 1.

Effect of vitamin supplements on serum level of vitamins

Individual serum levels of Vitamins D, B9, and B12 were significantly low in diabetic group compared to control. Interestingly, our findings demonstrated that cosupplementation with Vitamin D, B9, and B12 restored their concentrations back again to levels of the control group. There is also a significant increase in serum levels of the three vitamins as compared to the diabetic group, Table 2.

Effect of vitamin supplements on aortic tissue MDA and NO levels

Aortic tissue MDA level was significantly high in diabetic group with reduced NO level as compared to control group, after the administration of vitamin supplement whether individually or combined, we observed a significant reduction in aortic tissue MDA level and a significant increase in NO level compared to the diabetic group. Furthermore, no significant difference between aortic tissue MDA levels in diabetic group received Vitamins D, B9, and B12 and control. On the positive side, aortic tissue NO level was significantly high in the diabetic group received Vitamins D, B9, and B12 compared with diabetic received Vitamin D and diabetic received B9 and B12, Table 3.

Table 1: Serum levels of FBS and lipid profile in different groups

Groups	F.B.S (mg/dl)	Cholesterol (mg/dl)	T.G (mg/dl)	LDL (mg/dl)
Control	66.0±1.3	42.5±1.8	107.0±9.4	56.7±2.6
Diabetic	258.0±12.4[a]	160.6±8.6 [a]	257.5±8.5 [a]	113.7±6.8[a]
Diabetic received Vitamin D	173.6±4.9[abc]	88.75±11.7[ab]	146.0±5.0 [b]	79.0±4.8[ab]
Diabetic received Vitamins B9 and B12	185.0±8.0[abc]	94.7±9.0[abc]	153.7±15.9 [b]	91.0±6.4[abc]
Diabetic received Vitamins D, B_9, and B_{12}	138.7±5.5[ab]	62.5±6.2[b]	120.0±21.2[b]	73.0±4.6[ab]

Data are expressed as mean±SE. Significant: $P<0.05$. [a]Significant versus control group. [b]Significant versus diabetic group. [c]Significant versus diabetic received Vitamins D, B_9, and B_{12}. FBS: Fasting blood sugar, LDL: Low- density lipoprotein. N=10 in each group

Table 2: Serum levels of vitamins D, B_9, and B_{12} in different groups

Groups	Serum Vitamin D (ng/ml)	Serum Vitamin B_9 (ng/ml)	Serum Vitamin B_{12} (pmol/L)
Control	340.5±13.7	9.4±0.18	512.1±8.2
Diabetic	211.5±15.8[a]	3.7±0.3 [a]	233.0±13.8 [a]
Diabetic received Vitamin D	331.3±12.5[b]	4.3±0.2 [b]	267.2±8.7 [b]
Diabetic received Vitamins B_9 and B_{12}	242.8±9.8[ab]	8.9±0.3 [ab]	479.3±12.1[ab]
Diabetic received Vitamins D, B_9, and B_{12}	341.3±15.1[b]	8.8±0.5 [b]	474.5±15.0 [b]

Data are expressed as mean± SE. Significant: $P<0.05$. [a]Significant versus control group. [b]Significant versus diabetic group. [c]Significant versus diabetic received Vitamins D, B_9, and B12. N=10 in each group

Table 3: Levels of MDA and NO in aorta tissue samples of different groups

Groups	MDA (µmol/g tissue)	NO (µmol/g tissue)
Control	121.2±7.6	24.7±0.7
Diabetic	286.0±20.8[a]	10.5±0.4[a]
Diabetic received Vitamin D	169.0±13.1[ab]	17.8±0.6[abc]
Diabetic received Vitamins B_9 and B_{12}	165.0±15.3[ab]	17.2±0.8[abc]
Diabetic received Vitamins D, B_9, and B_{12}	148.7±5.1 [b]	20.4±0.8[ab]

Data are expressed as mean±SE. Significant: $P<0.05$. [a]Significant versus control group. [b]Significant versus diabetic group. [c]Significant versus diabetic received Vitamins D, B_9, and B_{12}. MDA: Malondialdehyde, NO: Nitric oxide. N=10 in each group

Table 4: Serum level of Hcy in different groups

Groups	Hcy (µmol/L)
Control	3.4±0.2
Diabetic	13.8±0.5[a]
Diabetic received Vitamin D	6.8±0.5[abc]
Diabetic received Vitamins B_9 and B_{12}	7.6±0.6[abc]
Diabetic received Vitamins D, B_9, and B_{12}	3.9±0.4[b]

Data are expressed as mean±SE. Significant: $P<0.05$. [a]Significant versus control group. [b]Significant versus diabetic group. [c]Significant versus diabetic received Vitamins D, B_9, and B12. Hcy : Homocysteine N=10 in each group

Effect of vitamin supplements on serum Hcy

Serum Hcy level was significantly high in diabetic group compared to other experimental groups. At the end of experiment, Hcy level was significantly low in the groups received vitamin supplements, but the reduction is more in the group received combination of the three vitamins, Table 4.

DISCUSSION

In the present study, a single subcutaneous dose of STZ-induced rapid elevation of blood sugar with sequencing adverse effect. Our results revealed that diabetic group received Vitamin D showed a significant reduction in FBS as Vitamin D activates β-cell endopeptidases dependent on calcium which induces β-cells to secrete insulin by increases in intracellular calcium concentration or by mediating β-cell calcium-dependent activation to facilitate the conversion of proinsulin to insulin [24]. Vitamin D also inhibits the production of IFN-γ and IL-2 cytokines, which activate macrophages that destruct the pancreatic islet cells. Notwithstanding, the diabetic group received Vitamins B9 and B12 showed significantly low levels of FBS compared to diabetic as B9 and B12 are cofactors for reactions involved in the formation of the important methyl donor S-adenosyl methionine that may lead to epigenetic modifications that decrease insulin resistant as reported previously [25].

It was reported that Vitamin D might delay the progression of atherogenesis and its deficiency indirectly contributes to the development of diabetes mellitus and dyslipidemia [26]. We observed that Vitamin D supplements produced a significant reduction in the levels of lipid profile; these are in line with a previous study, which revealed that Vitamin D has antiatherosclerotic effects through inhibition lipid peroxidation and attenuation of the inflammatory process of atherosclerosis [27]. There was a reduction in the lipid profile levels in the diabetic group received B9 and B12 compared to the diabetic group, this assisted with lowering of Hcy level [28], as high levels of Hcy activate 3-hydroxy-3-methylglutaryl coenzyme A reductase, which has a key role in cholesterol biosynthesis [29]. On the other hand, the diabetic group received Vitamins D, B9, and B12 showed the conspicuous reduction of levels of lipid profile than diabetic group received Vitamin D and the diabetic group received B9 and B12 only.

There was a significantly high level of serum Hcy and aortic tissue MDA but low level of aortic tissue NO in the diabetic group. These results are commensurate with previous studies showed that hyperhomocysteinemia associated with endothelial dysfunction due to oxidative stress [30] involved inflammation [31] increased the level of asymmetric dimethylarginine [32] which can result in decreasing endothelium-derived NO concentration and bioavailability [33]. Furthermore, MDA associated with the initiation and increase of reactive species that represent the main targets for inflammatory cells.

With reference to diabetic group received Vitamin D, a significant reduction of serum Hcy, aortic tissue MDA levels but high levels of tissue NO observed when compared to the diabetic group. The high levels of aortic tissue NO are in agreement with Ellam *et al*. who reported a fast increase in endothelial NO production through interaction with Vitamin D receptor after calcitriol supplement [34].

Equally, Vitamin D may modulate Hcy metabolism and can affect its serum concentration [35]. Nonetheless, Vitamin D inhibits the intracellular formation of advanced glycation end products which increase in diabetes and causes endothelial dysfunction [2]. In diabetic group received B9 and B12, the present findings showed that Hcy levels are significantly low compared to the diabetic group, as Vitamin B9 and B12 act as a cofactor for the enzyme methionine synthase that converts Hcy to methionine. Hcy is an independent risk factor for cardiovascular and thrombotic diseases and causes atherosclerosis as it leads to oxidative damage to endothelial, proliferation of smooth muscle, and lipid peroxidation [36].

Significant high levels of NO observed in the diabetic group received B9 and B12 compared to the diabetic group, underpinned by Caruso *et al*. earlier study [37]. Folic acid (Vitamin B9) and its active metabolite 5-methyltetrahydrofolate are directly scavenging superoxide radicals and improving NO bioavailability by increasing endothelial NO synthase coupling and NO production [38].

Outstandingly, the diabetic group received Vitamins D, B9, and B12 showed the most pronounced reduction of Hcy levels as compared to other diabetic groups received Vitamin D or received B9 and B12 only.

CONCLUSION

We concluded that coadministration of Vitamins D, B_9, and B_{12} exerted some antiatherosclerotic effects through inhibiting lipid peroxidation coupled with increasing aortic tissue NO. These findings may have important uses and implications in the modulation and protection against diabetic endothelial dysfunction.

AUTHOR'S CONTRIBUTIONS

Dr. Fakhria Al- Joufi and Dr. Mona Anwar designed the study and wrote the manuscript, Dr. Mona A. El- Bana performed biochemical assessment and statistical analysis. Dr. Ihab Tewfik contributed to the writing, amending, and approving of manuscript. All authors read and approved the final version.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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