Efficacy of Cosupplementation Therapy with Vitamins B₉, B₁₂, and D on Endothelial Dysfunction in Streptozotocin-Induced Diabetic Rats

FAKHRIA AL-JOUFI¹, MONA A EL-BANA², IHAB TEWFIK², MONA ANWAR¹*⁴

¹Department of Pharmacology, Ajloun University, Sakaka, KSA. ²Department of Medical Biochemistry, National Research Centre, Giza, Egypt. ³Division of Food, Nutrition and Public Health, University of Westminster, London W1W 6UW, United Kingdom. ⁴Research on Children with Special Needs Department, National Research Centre, Giza, Egypt. Email: mona_anwar2@yahoo.com

ABSTRACT

Objective: This study evaluated the effects of Vitamins D, B₉, and B₁₂, 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 B₉ and B₁₂, and diabetic received Vitamins B₉, B₁₂, 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 B₉, B₁₂, and D.

Results: Diabetic rat received Vitamin D and diabetic rat received Vitamins B₉ and B₁₂ 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 B₉, B₁₂, 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 B₁₂.
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, 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.

**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 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.

**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         | 6.0±1.3       | 42.5±1.8            | 107.0±9.4   | 56.7±2.6    |
| Diabetic        | 258.0±12.4    | 160.6±8.6           | 257.5±8.5   | 113.7±6.8   |
| Diabetic received Vitamin D | 173.6±4.9a   | 88.75±1.7a          | 146.0±5.0a  | 79.0±4.8a   |
| Diabetic received Vitamins B9 and B12 | 185.0±8.0a    | 94.7±9.0a          | 153.7±15.9b | 91.0±5.4a    |
| Diabetic received Vitamins D, B9, and B12 | 138.7±5.5a    | 62.5±6.2a          | 120.0±21.2b | 73.0±4.6a    |

Data are expressed as mean±SE. Significant: *p<0.05 versus control group. **Significant versus diabetic group. "Significant versus diabetic received Vitamin D, B9 and B12. FBS: Fasting blood sugar; LDL: Low-density lipoprotein. N=10 in each group.

**Table 2:** Serum levels of vitamins D, B9, and B12 in different groups

| Groups          | Serum Vitamin D (ng/ml) | Serum Vitamin B9 (ng/ml) | Serum Vitamin B12 (pmol/L) |
|-----------------|-------------------------|-------------------------|---------------------------|
| Control         | 340.5±13.7              | 9.4±0.18                | 512.1±8.2                 |
| Diabetic        | 211.5±15.8              | 3.7±0.3                 | 233.0±13.8                |
| Diabetic received Vitamin D | 331.3±12.5a | 4.3±0.2a               | 267.2±8.7a                |
| Diabetic received Vitamins B9 and B12 | 242.9±9.8a | 8.9±0.3a               | 479.3±12.1a               |
| Diabetic received Vitamins D, B9, and B12 | 341.3±15.1a | 8.8±0.5b               | 474.5±15.0b               |

Data are expressed as mean±SE. Significant: *p<0.05 versus control group. **Significant versus diabetic group. "Significant versus diabetic received Vitamin D, B9 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<sup>a</sup> | 10.5±0.4<sup>a</sup> |
| Diabetic received Vitamin D   | 169.0±13.1<sup>b</sup> | 17.8±0.6<sup>b</sup> |
| Diabetic received Vitamins B<sub>9</sub> and B<sub>12</sub> | 16.5±15.3<sup>c</sup> | 17.2±0.9<sup>c</sup> |
| Diabetic received Vitamins D, B<sub>9</sub> and B<sub>12</sub> | 14.8±5.1<sup>c</sup> | 20.4±0.8<sup>c</sup> |

Data are expressed as mean±SE. Significant: P<0.05. Significant versus control group. Significant versus diabetic received Vitamin D, B<sub>9</sub> and B<sub>12</sub>. 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<sup>a</sup> |
| Diabetic received Vitamin D   | 6.0±0.5<sup>ab</sup> |
| Diabetic received Vitamins B<sub>9</sub> and B<sub>12</sub> | 7.6±0.6<sup>abc</sup> |
| Diabetic received Vitamins D, B<sub>9</sub> and B<sub>12</sub> | 3.9±0.4<sup>c</sup> |

Data are expressed as mean±SE. Significant: P<0.05. Significant versus control group. Significant versus diabetic received Vitamins D, B<sub>9</sub> and B<sub>12</sub>. 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 B<sub>9</sub> and B<sub>12</sub> showed significantly low levels of FBS compared to diabetic as B<sub>9</sub> and B<sub>12</sub> 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 atherosclerosis 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 B<sub>9</sub> and B<sub>12</sub> 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, B<sub>9</sub>, and B<sub>12</sub> showed the conspicuous reduction of levels of lipid profile than diabetic group received Vitamin D and the diabetic group received B<sub>9</sub> and B<sub>12</sub> 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 B<sub>9</sub> and B<sub>12</sub>, the present findings showed that Hcy levels are significantly low compared to the diabetic group, as Vitamin B<sub>9</sub> and B<sub>12</sub> act as a cofactor for the enzymehomocysteine 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 B<sub>9</sub> and B<sub>12</sub> compared to the diabetic group, underpinned by Caruso et al. earlier study [37]. Folic acid (Vitamin B<sub>9</sub>) 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, B<sub>9</sub>, and B<sub>12</sub> showed the most pronounced reduction of Hcy levels as compared to other diabetic groups received Vitamin D or received B<sub>9</sub> and B<sub>12</sub> only.

CONCLUSION

We concluded that coadministration of Vitamins D, B<sub>9</sub>, and B<sub>12</sub> 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.

REFERENCES

1. Hamilton SJ, Watts GF. Endothelial dysfunction in diabetes: Pathogenesis, significance, and treatment. Rev Diabet Stud 2013;10:133-56.
2. Sena CM, Pereira AM, Seiça R. Endothelial dysfunction - a major mediator of diabetic vascular disease. Biochim Biophys Acta 2013;1832:2216-31.

3. Wang Z, Li AY, Guo QH, Zhang JP, An Q, Guo YJ, et al. Effects of cyclic intermittent hypoxia on ET-1 responsiveness and endothelial dysfunction of pulmonary arteries in rats. PLoS One 2013;8:e58078.

4. Boucher BJ. Vitamin D insufficiency and diabetes risks. Curr Drug Targets 2011;12:61-87.

5. Kolnir A, Uberti F, Grossini E, Vacca G, Carda S, Invernizzi M, et al. 1α,25-dihydroxycholecalciferol induces nitric oxide production in cultured endothelial cells. Cell Physiol Biochem 2011;27:661-8.

6. Polidoro L, Properzi G, Marampon F, Gravina GL, Festuccia C, Di Cesare E, et al. Vitamin D protects human endothelial cells from H₂O₂ oxidant injury through the erk/eri-1 axis activation. J Cardiovasc Transl Res 2013;6:221-31.

7. Uberti F, Lattuada D, Morsanuto V, Nava U, Bolis G, Vacca G, et al. Vitamin D protects human endothelial cells from oxidative stress through the autophagic and survival pathways. J Clin Endocrinol Metab 2014;99:1367-74.

8. Hussein J, El-Khayat Z, Morsy S, Oraby F, Singh Y. The effect of fish oil on oxidant/antioxidant status in diabetic rats through the reduction of arachidonic acid in the cell membrane. Int J Pharm Pharm Sci 2014;6:196-9.

9. Bulyonko VA, O'Malley BW. Nuclear receptor coactivators: Structural and functional biochemistry. Biochemistry 2011;50:313-28.

10. Prosser DE, Jones G. Enzymes involved in the activation and inactivation of vitamin D. Trends Biochem Sci 2004;29:649-54.

11. Böke DD. Extra renal synthesis of 1,25 dihydroxy vitamin D and its health implications. Clin Rev Bone Miner Metab 2009;7:114-25.

12. Hussein J, El-Khayat Z, Mofidy M. Study of arachidonic acid releasing status in diabetic rats treated with flaxseed oil. Int J Pharm Pharm Sci 2016;8:975-1491.

13. Kohli R, Meininga CJ, Haynes TE, Yan W, Self JT, Wu G, et al. Dietary L-arginine supplementation enhances endothelial nitric oxide synthesis in streptozotocin-induced diabetic rats. J Nutr 2004;134:600-8.

14. Koleganova N, Piecha G, Ritz E, Gross ML. Calcitriol ameliorates capillary deficit and fibrosis of the heart in subtotally nephrectomized rats. J Nephrol Transplant 2009;24:779-87.

15. Deniz ÖG, Kivrak EG, Kaplan AA, Altunkaya N, Kiziltas M. Effects of folic acid on rat kidney exposed to 900 MHz electromagnetic radiation. J Microsc Ultrastruct 2017;5:198-205.

16. Ekaidem IS, Akpanabiatu MI, Uboh FE, Eka OU. Effect of folic acid and vitamin B(12) administration on phenytoin induced toxicity in rats. Indian J Clin Biochem 2007;22:36-40.

17. Passing H, Bablok W. A new biometrical procedure for testing the equality of measurements from two different analytical methods. Application of linear regression procedures for method comparison studies in clinical chemistry, part I. J Clin Chem Clin Biochem 1983;21:709-20.

18. Allain CC, Poon LS, Chan CS, Richmond W, Fu PC. Enzymatic determination of total serum cholesterol. Clin Chem 1974;20:470-5.

19. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin Chem 1972;18:499-502.

20. Glick MR, Ryder KW, Jackson SA. Graphical comparisons of interferences in clinical chemistry instrumentation. Clin Chem 1986;32:470-5.

21. Hussein J, El-Khayat Z, Abdel Latif Y, Medhat D, Morsy S, Oraby F. Evaluation of brain monoamines in diabetic rats treated with quercetin. J Pharm Clin Res 2014;6:384-90.

22. Tatsch E, Bochi OV, Pereira Rda S, Kober H, Agertt VA, de Campos MM, et al. A simple and inexpensive automated technique for measurement of serum nitrate/nitrite. Clin Biochem 2011;44:348-50.

23. Ruiz-Larrea MB, Leal AM, Liza M, Lacort M, de Groot H. Antioxidant effects of estradiol and 2-hydroxyestradiol on iron-induced lipid peroxidation of rat liver microsomes. Steroids 1994;59:383-8.

24. Mitri J, Pittas AG. Vitamin D and diabetes. Endocrino Hel Metab Clin North Am 2014;43:205-32.

25. Sinclair KD, Allegretti C, Singh R, Gardner DS, Sebastian S, Bispinck H, et al. DNA methylation, insulin resistance, and blood pressure in offspring determined by maternal periconceptional B vitamin and methionine status. Proc Natl Acad Sci U S A 2007;104:19351-6.

26. Mertens PR, Muller RM. Vitamin D and cardiovascular risk. Int Urol Nephrol 2010;42:165-71.

27. Malek HA, Sluta A. Effect of a high dose of vitamin D on a rabbit model of atherosclerosis. Int J Immunopharmacol Pharmacol 2014;7:195-201.

28. Antoniades C, Antonopoulos AS, Tousoulis D, Marinou K, Stefanadis C. Homocysteine and coronary atherosclerosis: From folate fortification to the recent clinical trials. Eur Heart J 2009;30:6-15.

29. Bhargava S, Ali A, Bhargava EK, Manocha A, Kankra M, Das S, et al. Lowering homocysteine and modifying nutritional status with folic acid and vitamin B(12) in Indian patients of vascular disease. J Clin Biochem Nutr 2012;50:222-6.

30. Hoffmann M. Hypothesis: Hyperhomocysteinemia is an indicator of oxidant stress. Med Hypotheses 2011;77:1088-93.

31. Arzamastsev DD, Karpenko AA, Kostichchenko GI. Inflammation of the vascular wall and hyperhomocysteinemia in patients with atherosclerosis obliterans of lower limb arteries. Angiol Sovsd Khir 2012;18:27-30.

32. Magné J, Heneau JF, Borderie D, Mathé V, Bos C, Mariotti F, et al. Plasma asymmetric and symmetric dimethylarginine in a rat model of endothelial dysfunction induced by acute hyperhomocysteinemia. Amino Acids 2015;47:1975-82.

33. Emekszis HC, Serdaroglu A, Biberoglu G, Gulbahar O, Arhan E, Cansu A, et al. Assessment of atherosclerosis risk due to the homocysteine-asymmetric dimethylarginine-nitric oxide cascade in children taking antiepileptic drugs. Seizure 2013;22:124-7.

34. Illam T, Hameed A, ul Haque R, Muthana M, Wilkie M, Francis SE, et al. Vitamin D deficiency and exogenous vitamin D excess similarly increase diffuse atherosclerotic calcification in apolipoprotein E knockout mice. PLoS One 2014;9:e88767.

35. Kriebitzsch C, Verlinden L, Eelen G, van Schoor NM, Swart K, Lips P, et al. 1,25-dihydroxyvitan D3 influences cellular homocysteine levels in murine preosteoblastic MC3T3-E1 cells by direct regulation of cystathionine β-synthase. PLoS One 2014;9:e88767.

36. Nursaltim A, Siregar P, Widyahening IS. Effect of folic acid, vitamin B6 and vitamin B12 supplementation on phenytoin induced toxicity in rats. Asian J Pharm Clin Res 2011;4:97-110.

37. Stanlewicz AE, Kenney WL. Role of folic acid in nitric oxide bioavailability and vascular endothelial function. Nutr Rev 2017;75:61-70.