

Original Research Article

The Protective Effect of Vitamin D on Cerebral Infarction in Rats Received High Fructose Diet

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Abstract

Background and aim: The relationship between insulin resistance (IR), hypovitamin D and cerebral infarction and its exact mechanism are not fully understood. However, oxidative stress and pro-inflammatory mediators may be involved. Thus, the aim of the present study was to investigate the effects of vitamin D on cerebral infarction, insulin resistance and inflammatory mediators in rats received high-fructose-diet.

Material and methods: Eight four adult albino rats were divided randomly into 3 groups, normal control group, diabetic group rats received high fructose diet for 2 months without no treatment, diabetic rats received alphacalcidol (10µg/kg/day, orally), which was continued daily throughout the experiment, After 2 months, fasting blood glucose level and insulin, IR was evaluated by the homeostasis model assessment method (HOMA-IR). Some cerebrovascular risk markers as lipid profile (total cholesterol, HDL-C and triglycerides), as well as inflammatory biomarker interleukin-6 and cerebral infarction size were measured.

Results: Rats had high fructose diet showed low 1, 25 (OH)₂ D, with a significant (p <0.05) increase in fasting blood glucose level and higher HOMA-IR index, total cholesterol, low-density lipoprotein cholesterol (LDL-C), triglyceride and IL-6 as compared to control group. There were significant correlations between 1, 25 (OH)₂ D levels and HOMA-IR index (r=-0.47; p <0.01), 1, 25 (OH)₂ D, total cholesterol (r=-0.34; p <0.01); HDL-C (r=0.54; p <0.01), LDL-C (r=-0.34; P <0.01), IL-6 (r=-0.33; p <0.05). A two month oral alphacalcidol (10 ug/kg/day, orally) treatment markedly decreased HOMA-IR index (p <0.001), LDL, triglycerides and IL-6 and significantly reduced total cholesterol and cerebral infarction size.

Conclusions: Our data showed that, low 1, 25(OH)₂ D₃ values are related to IR and associated with chronic inflammatory state and dyslipidemia, all of them can increase cerebrovascular risk. Vitamin D analogue significantly ameliorated the deleterious biochemical impact of diabetes mellitus protects against cerebral injury by its anti-inflammatory and decreasing insulin resistance in diabetic rats.

Key words

Vitamin D, Insulin Resistance, Cerebrovascular stroke, Inflammatory markers, Oxidative stress.

Introduction

Cerebrovascular stroke, after coronary heart diseases and cancer, is the third common cause of death in most countries, and each year there is approximately 500,000 cases of stroke in the United States, with 175,000 fatalities from the cause [1].

Diabetes mellitus is a major independent risk factor for stroke, and hyperglycemia is associated with poor outcome because of increase the oxidative stress and inflammation [2].

Oxidative stress and inflammation are responsible for neuronal damage in acute cerebral ischemia. In ischemia–reperfusion injury, reactive oxygen and nitrogen species induce protein oxidation, DNA damage, and lipid peroxidation [3]. Oxidative damage induced by reactive oxygen radicals causes complex interactions with inflammation and apoptosis-like cell death, and the results expand brain damage [4].

Among known cerebrovascular risk factors are dyslipidemia with elevated levels of triglycerides and low density lipoprotein-cholesterol (LDL-C) and lower level of high density lipoprotein-cholesterol (HDL-C) [5, 6]. Other risk markers studied in the pathogenesis of cerebrovascular diseases is the high levels of inflammatory mediators such as interleukin-6 (IL-6) [7] tumor necrosis factor alpha (TNF- α) [8] and nitric oxide [9] that can predict cerebral risk.

A growing body of evidence supports an association between vitamin D and cerebrovascular disease. However, the mechanisms underlying this association are

unknown. Low vitamin D levels are predictive for strokes in humans, whereas vitamin D supplements are associated with decreased risk of all-cause mortality [10].

In this study, it was hypothesized that low vitamin D status is a contributing factor in the pathophysiology of insulin resistance and cerebrovascular risk found in diabetic patients. To examine this hypothesis, we examine the effects of administration of Vitamin D in a model of high fructose diet induced type 2 diabetic rats. Determine the relationship between vitamin D status with insulin resistance and some cerebrovascular risk factors as lipid profile and the inflammatory biomarkers; IL-6. Test whether vitamin D supplementation can improve insulin sensitivity and reduce cerebrovascular risk in diabetic groups.

Materials and methods

Experimental Protocol

Animal used

Adult white male albino-rats, (4 weeks old) were brought from Experimental Animal Breeding Farm, Helwan. All animals were housed in controlled laboratory condition at 20-25°C in a 12 hours light/dark cycle and had free access to food and water. They have acclimatized for one week and were caged (6 per cage) in fully ventilated room (at room temperature) in Pharmacology Department, Benha Faculty of Medicine. The experimental protocol met the national guiding on the proper care and use of animals in the laboratory research and an institutional animal ethics committee approved the study.

Experimental design

The rats were randomly divided into three groups.

Group I: sham control, animals (n= 12): rats fed standard rodent chow and water throughout the experimental period.

Group II: diabetic group (n=18): rats were fed with high fructose diet (65% of diet) for 2 months (Douard et al., 2014).

Group III: diabetic rats were treated with Vitamin D analogue (n=18): rats were received alphacalcidol (Sigma, St. Louis, MO), (10 µg/kg body weight/day/orally) for a period of 2 months before middle cerebral artery occlusion (MCAo) [11].

All animal groups undergo focal cerebral ischemia by the occlusion of the right middle cerebral artery as previously described [12].

Each group was subjected to the following evaluations.

Biochemical assessment

At the end of the experiment, blood samples were collected from the retro-bulbar sinus of rat's eye by using heparinized capillary tubes [13]. Two ml of blood were delivered in clean, dry test tubes and allowed to clot at room temperature. The serum was separated by centrifugation at 2000 rounds/minute for 10 minutes. Sera were kept in tightly closed vials at 20°C until used to measure serum level of vitamin D, blood glucose, insulin resistance, lipid profile and inflammatory markers.

Vitamin D level

ELISA method was performed by using specific kits measuring 1, 25(OH)₂ vitamin D and 25-hydroxy vitamin D (IBL International, Germany, Cat No. UK51081) [14, 15].

Serum blood glucose level and insulin resistance

The serum concentration of glucose was measured by enzymatic colorimetric glucose assay kit (Diamond, D-P international, Germany) [16]. Insulin level was determined by Enzyme-linked immunosorbent assay (ELISA) kits (Immunospec Corporation, CA, E 29-072, and

USA) [17]. Insulin resistance was assessed using the homeostasis model assessment of insulin resistance (HOMA-IR) originally described by Matheu et al. [18] by the following formula: $HOMA-IR = \frac{\text{fasting blood glucose (mmol/L)} \times \text{fasting insulin (}\mu\text{U/L)}}{22.5}$. Rats were considered to have IR if $HOMA-IR \geq 2.6$ [19].

Total cholesterol, HDL-C and Triglycerides levels

Quantitative enzymatic colorimetric determination was used for measuring total cholesterol [20], HDL-C levels [21] and triglycerides [22]. This was performed by using kits supplied by bio Merieux, France Cat. No. 61. 225, bio Merieux, France Cat. No.61. 236 and Stanbio-HDL-C kit, procedure No. 0599 respectively. LDL-C levels were calculated using the following formula: $LDL-C = \frac{\text{Total cholesterol} - \text{Triglyceride}}{5} - \text{HDL} - C$ [23].

Serum content of IL-6

Estimation of IL-6 by ELISA technique using (Ray Bio ® Mouse IL-6)

By following the manufacture instruction according the protocol of Howord and O'Gara, [24].

Determination of the infarct size

The rats in all groups were sacrificed, and the brains were removed and sectioned in coronal planes 3.0 and 5.0 mm from the frontal pole. The middle segment were placed in a phosphate buffer solution (pH: 7.4) containing 2% 2, 3, 5 triphenyl tetrazolium hydrochloride (TTC) and incubated at 37°C thirty minutes. Viable brain appears brick red while infarcted area appears brownish [25]. Clear glass were placed over both sides of each slides. Non stained areas were carefully traced on clear plastic sheets and measured. The infarct size was calculated as percentage of inverted area to whole cerebral cortex surface area.

Statistical analysis

Multiple comparisons were performed using one-way Anova analysis of variance (ANOVA)

followed by tukey's test as a post-hoc test. The 0.05 level of probability was used as the criterion for significance. All statistical analyses were performed using GraphPad Istat version software package. Statistical Analysis results are presented as mean \pm standard error (mean \pm SEM) [26, 27].

Results

Fructose diet induced changes in rats

High fructose diet administration significantly reduced serum 1, 25 (OH)₂ vitamin D and 25 – hydroxy vitamin D. On the other hand, fasting

blood glucose level and fasting insulin were significantly higher in compare with control group. Insulin resistance in rats with low 1, 25 (OH)₂ D was significantly higher (i.e., HOMA-IR) compared with control group. Moreover, there were significant higher levels of total cholesterol, LDL-C, level of triglycerides with significant lower levels of HDL-C in compared with normal control (**Table - 1**). Moreover, the serum IL-6 showed significant higher level in rats with low 1, 25 (OH)₂ D compared to normal control (**Table - 2**).

Table - 1: Effect of vitamin D administration on Lipid profile and inflammatory biomarker IL-6 of all rats groups after 2 months alphacalcidol supplementation.

Groups Parameters	Control group	Diabetic group treatment	Infarcted without	MCAO + alphacalcidol supplementation
Total cholesterol (mg/dL)	172.27 \pm 12.8	225.30 \pm 11*		190.5 \pm 13.5#
HDL-C (mg/dL)	56.4 \pm 3.90	41.76 \pm 3.62*		50.41 \pm 3.41#
Triglycerides (mg/dL)	102.67 \pm 6.72	149.55 \pm 12.15*		144.60 \pm 11.71
LDL-C (mg/dL)	95.33 \pm 8.67	153.68 \pm 11.88*		136.18 \pm 9.18#
IL-6 (pg/mL)	3.79 \pm 0.11	7.46 \pm 0.31*		5.78 \pm 0.2

Data are expressed as mean \pm S.E.; HDL-C, high-density lipoprotein cholesterol ; LDL-C, low-density lipoprotein cholesterol; IL-6, interleukin-6. *Statistically significant vs. control group. # statistically significant vs. values of non-treated group (unpaired t-test).

Table - 2: Effects of vitamin D administration on biochemical characteristics of all groups and after 2 months alphacalcidol supplementation.

Groups Parameters	Control	Diabetic group without treatment	Infarcted	MCAO+ alphacalcidol supplementation
1, 25(OH) ₂ D (pg/mL)	39.27 \pm 2.54	22.97 \pm 1.48*		25.07 \pm 0.65
25 (OH) D	41 \pm 2.36	26.57 \pm 1.36		27.91 \pm 0.91
Insulin (μ U/ml)	8.21 \pm 0.33	13.61 \pm 0.17*		10.67 \pm 0.68#
Glucose (mmol/L)	4.99 \pm 0.03	7.14 \pm 0.46*		5.61 \pm 0.42
HOMA-IR index (mmol/L - μ U/ml)	1.88 \pm 0.01	3.78 \pm 0.02*		2.92 \pm 0.03#

Data are presented as mean \pm S.E. and analyzed by One Way ANOVA using Tuckey Kramer post-test for comparison at P<0.05. 1, 25-dihydroxyvitamin D; HOMA- IR, homeostasis model assessment of insulin resistance; IR, insulin resistance. * Statistically significant vs. control group. # statistically significant vs. non-treated group (unpaired t-test).

Pearson correlation coefficient analyses revealed that levels of 25 (OH) D was significantly and negatively correlated with HOMA-IR index ($r = -0.47$; $p < 0.01$) (**Figure - 1**). Serum levels of 25-hydroxy vitamin D was significantly and negatively correlated with total cholesterol ($r = -0.34$; $p < 0.01$), LDL-C ($r = -0.62$; $P < 0.01$), significantly and positively correlated with HDL-C and inflammatory marker IL-6 ($r = -0.33$; $p < 0.05$) ($r = 0.51$; $p < 0.01$) (**Figure - 2a, 2b, 2c, 2d** respectively). On the other hand, 1, 25 (OH)₂ D was not related to triglyceride levels.

Figure - 1: Correlation between 25-hydroxyvitamin D and HOMA-IR index.

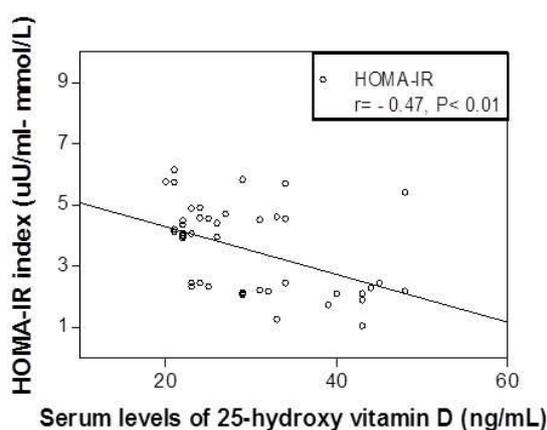
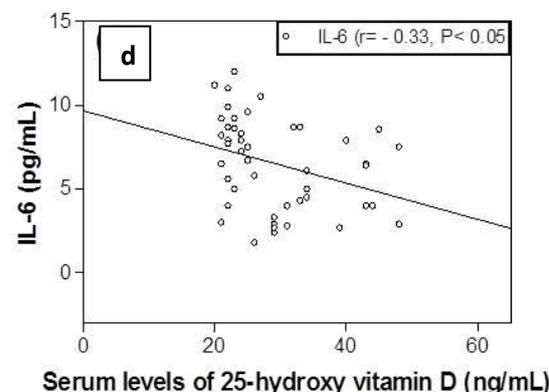
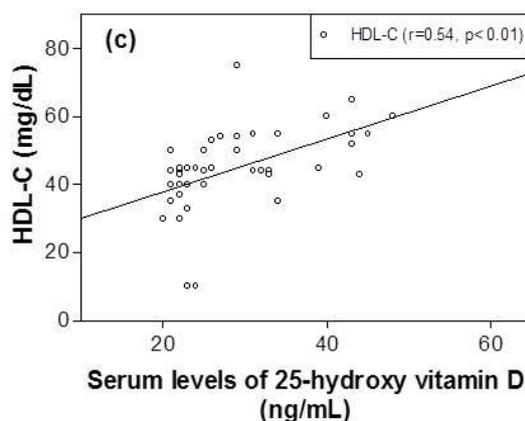
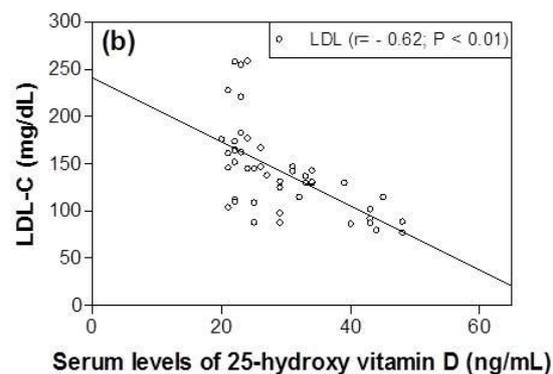
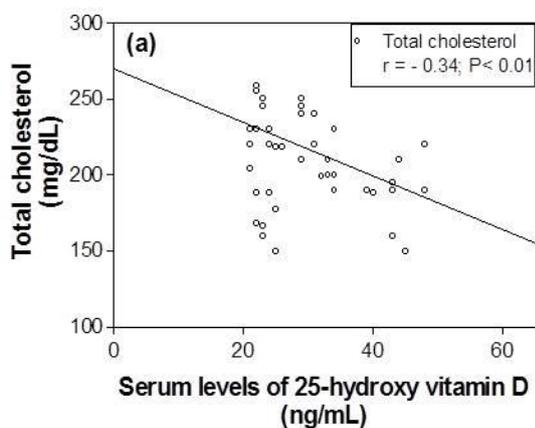


Figure - 2: Correlation between 25-hydroxyvitamin D and total cholesterol (a), low-density lipoprotein cholesterol (LDL-C) (b), and high-density lipoprotein cholesterol (HDL-C) (c), and interleukin-6 (IL-6) (d) in the diabetic rats.



Effects of administration of vitamin D on high fructose diet induced changes

After 2 month oral alphacalcidol supplementation for rats with low levels of 1, 25 (OH)₂ vitamin D we observed that vitamin D analogue supplementation resulted in significant decrease in insulin resistance i.e. reduced HOMA-IR index (2.92 ± 1.3 versus 3.78 ± 1.12) with significant decline in serum levels of insulin. At the end of supplementation period lipid bioassay showed a significant reduction of

total cholesterol, LDL-C ($P < 0.05$) and a slight non-significant decrease in triglyceride, while HDL-C was significantly raised as compared to the values before treatment. Additionally there was a significant reduction in the inflammatory biomarker; IL-6.

Effect of administration of vitamin D on cerebral infarction size:

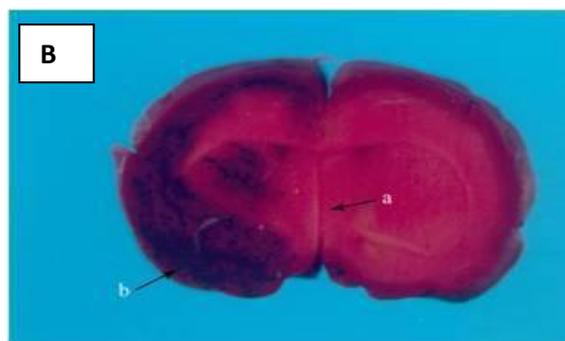
Results of the present work showed that, the cerebral infarct size was smaller in rats supplemented with vitamin D than in infarct group without treatment (Table - 3, Figure – 3A, 3B, 3C).

Table - 3: Effects of vitamin D on the cerebral infarct size.

Groups	Normal Control group	Diabetic infarcted group without treatment	MCAO+ alpa calcidol supplement ation
Cerebral infarct size	0	47.53± 4.26 *	22.73± 1.2 #

Data are presented as mean± S.E. and analyzed by One Way ANOVA using Tuckey Kramer post-test for comparison at $P < 0.05$

Figure - 3: Triphenyl tetrazolium demonstrating that administration of 1, 25(OH)₂ Vitamin D3 for two months diminishes cortical infarction induced by MCA ligation. A) Normal control group, B) infarct group with no treatment, and C) 1, 25(OH)₂ Vitamin D3 treated group.



Discussion

The current study showed that vitamin D had neuroprotective effects against permanent cerebral ischemia injury. Two months administration of vitamin D analogue before middle cerebral artery occlusion significantly reduced infarct volume and resulted in reduction of pro-inflammatory mediators as IL-6.

In the present study the 1, 25(OH)₂ D rats showed lower insulin sensitivity, and high insulin resistance ($HOMA-IR \geq 2.6$) than normal control rats with normal 1, 25(OH)₂ D levels. The level of 25-hydroxy vitamin D was negatively correlated with HOMA-IR. These results were in line with several observational studies suggested that low vitamin D levels were associated with insulin resistance or impaired insulin secretion in diabetic rats [28, 29] and in healthy subjects [30, 31].

The role of low levels of 1, 25(OH)₂ D in decreasing insulin sensitivity in diabetic is that 1, 25(OH)₂ D may have a beneficial effect on insulin responsiveness by either stimulating the expression of insulin receptors [32] or regulating the homeostasis of calcium, essential for insulin-mediated intracellular processes in insulin

responsive tissues [28, 33]. The pancreas also possesses the vitamin D receptor and 1- α hydroxylase at which circulating 25 (OH) D can be converted there to 1,25 (OH)₂ D to work as a paracrine or autocrine hormone [33].

Our findings demonstrated also that rats with low 1, 25 (OH)₂ D had higher levels of inflammatory marker IL-6 than normal control group, the serum levels of those biomarker was negatively correlated with serum levels of 1, 25 (OH)₂ D. this indicate that lower 1,25 (OH)₂ D is associated with inflammatory state. Recent studies evaluating the anti-inflammatory properties of vitamin D found that vitamin D deficiency has been correlated with circulating markers of inflammation in diabetic rats [28].

Vitamin D receptor is present in several immune cells, such as monocytes, macrophages and activated T and B lymphocytes [19]. Vitamin D interferes with cytokine production of monocytes and lymphocytes [34] and down regulates the production of pro-inflammatory cytokines, particularly TNF- α and IL-6 [35, 36, 37].

Results of this study could explain, in part how vitamin D status can be implicated in the etiology of decrease insulin sensitivity in diabetic rats by the presence of inflammation with lower vitamin D levels. One of the hallmarks of insulin resistance is low-grade inflammation. High amounts of circulating inflammatory markers contribute significantly to insulin resistance in muscle and adipose tissue [38].

Another aim of our study was to examine the association between vitamin D status and the risk of cerebral infarction disease commonly seen in diabetic patients. Results of this study indicated cerebral infarction burden increased with low 1, 25(OH)₂ D levels. As diabetic rats with low 1, 25(OH)₂ D had increased risky lipids; total cholesterol, LDL-C together with increased levels of inflammatory biomarkers IL-6 compared with normal control group. In all diabetic rats the levels of 1, 25 (OH)₂ D showed negative correlations with risky lipids and

inflammatory biomarkers. Similar associations between vitamin D status and the increase cerebral infarction burden have been reported in previous literatures [39, 40].

Conclusion

From the data obtained in the present work it could be concluded that: type 2 diabetes induced by high fructose-diet could induce cerebrovascular complication as indicated by increased metabolic disturbances (insulin resistance, dyslipidemia, and increased pro-inflammatory mediators) as well as increase cerebral infarction size. Treatment with vitamin D resulted in prophylaxis against cerebral stroke induced by middle cerebral artery occlusion as showed by marked reductions in infarction size along with improvement of metabolic changes and decreased levels of IL-6 serum level.

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