Original Article

The Effect of 1, 25 Dihydroxy-Vitamin D3 on Insulin Sensitivity and Expression of JNK in Diabetes – Induced Liver Complications in Rats

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Abstract

Introduction: NAFLD one of most common complication of diabetes mellitus. *Several studies tried to understand its mechanism or to prevent it. The current study aim* is to study the possible effect of treatment of vitamin D on the expression of inflammatory cytokine C-Jun N-terminal kinase (JNK) in rat models of diabetic liver. **Methods:** *thirty male albino rats were assigned into two groups group I (healthy control) that were fed normal diet (n= 10), group II (n=20) received HFD for 8 week and then injected with STZ 30* mg/kg. *After detection of hyperglyceamia this group was randomly divided into subgroups, group IIA received vitamin D for 8 weeks in a dose of* (0.03 µg/kg/day), *and group IIB was kept without treatment.* **Results & Conclusion:** vitamin D significantly improves glucose intolerance, insulin sensitivity, decreased HOMA-IR, decreased serum ALT and decreased AST, increased SOD and decreased liver JNK expression.

Key Words: diabetic liver, NAFLD, JNK, 1,25dihydroxy vitamin D3, insulin sensitivity, SOD, Interleukine-1β **.**

Introduction

Diabetes mellitus affects people worldwide and poses major public health and socio-economic challenges.[(1)] Monitoring of blood glucose is essential in optimizing long-term outcomes towards reducing the co-morbid conditions that may arise in patients with T2DM[(2)]. High Fat Diet (HFD) has also been utilized to model chronic inflammation, which is an important pathogenic mechanism of T2DM. The HFD - mediated chronic inflammation is marked by increased TNF-α, IL-1β, and IL-6 in the circulation. [(3)]

T2DM is strongly associated with obesity and insulin resistance [(4)] as well as defects in pancreatic β-cell function and mass. Insulin is important in carbohydrate, fat and protein metabolism[(5)].

Insulin resistance acts as a key link between obesity and T2DM , It is likely that the development of obesity-induced insulin resistance involves a complex interplay of genetic and environmental factors.[(6)] The Insulin Receptor (IR) signaling pathway is triggered by its binding of the IR a receptor that belongs to tyrosine kinase receptor family. Hormone engagement stimulates the intrinsic tyrosine kinase activity of the IR resulting in the auto phosphorylation on the β subunit cytoplasmic tails. [(7)]

JNK: JNKs are serine threonine protein kinases that phosphorylate Jun family members, components of activator protein 1 (AP-1) transcription factors JNK also known as stress-activated Mitogen Protein Kinase (AMPK).[(8)]

Complication of diabetes: Chronic complications of diabetes which involve coronary artery disease, renal, liver and ophthalmologic diseases are the primary cause of disability and mortality in DM patients. It is reported that the standardized mortality rate from end-stage liver disease (i.e. cirrhosis) is higher than that for cardiovascular disease among patients with diabetes.[9]

Liver complications: NAFLD the most common chronic hepatopathy worldwide, reaching a prevalence of above 70% in patients with T2DM. [10] Inflammation is a crucial part of almost all acute and chronic liver disorders including (NAFLD).[11]

Interleukine-1β: IL-1β is a Pro-inflammatory cytokine not expressed in healthy liver. It acts via specific receptors involved in all inflammatory processes in the liver including regulation of insulin resistance and fibrosis. [12]

Oxidative stress is defined as an imbalance in the oxidant-to-antioxidant ratio, causing the generation of free radicals [13]. **Super oxide-dismutase (SOD):** SOD is a key antioxidant enzyme that dismutates superoxide anion (O_2-) to continuously form H_2O_2 and O_2 .[14]

Vitamin D: Vitamin D is a lipophilic molecule essential to maintain calcium and phosphate balance and osteometabolic system regulation. The steroid hormone Vitamin D (Vit D) can be produced by the body or obtained through the diet.[15] Evidence from various directions, including observational and experimental studies, indicates the protective capacity of 1,25-dihydroxyvitamin D3 (active vitamin D) in chronic low-intensity inflammation and IR in T2DM and its complications; although the underlying mechanism is yet to be clarified[16].

Aim of the Work

The purpose of the present study is to develop an appropriate stable animal model which is analogous to the human T2DM through a combination of high-fat diet with e low-dose STZ injection to test the hypothesis that vitamin D may improve the diabetes-induced liver complications by suppressing the expression of inflammatory cytokine and C-Jun N-terminal kinase (JNK) in the liver of this rat models of diabetes**.**

Materials & Methods

The study was conducted on thirty healthy male albino rats with body weight (100–150 g). Rats were housed in standard polypropylene cages (three rats/cage).[17] The period of experiment was 16 weeks. The Experiment was conducted in accordance with the guidelines set by the Research Ethics Committee of the Faculty of Medicine Alexandria University. After a week of acclimatization, rats were randomly divided into two groups:

Group (I): [(Control):(n=10)] rats in this group were fed a regular standard chow diet (60% carbohydrates,5% fat and 18% protein).[18]

Group(II): (n=20) rats of this group were fed a high fat diet HFD (22% fat, 48% carbohydrate, 20% protein) with a total caloric value of 44.3 kcal /kg. [17]:

After 8 weeks from the start of the diet regimen, all rats of group II received a single low dose of STZ 30 mg/kg (dissolved in 0.1 ml sodium citrate buffer at pH (4.4) intra peritoneal injection (IP) .[17]

Rats of the Control group (Group I) received a single IP injection of ml of sodium citrate buffer only. One week later, random blood glucose level of all rats was measured in blood samples collected from tail vein, using a glucometer.[19] Diabetes was confirmed by the detection of hyperglycemia (random non-fasting glucose level, >300mg/dl),[20] T2DM model rats (n=10) were randomly divided equally into 2 groups IIA and IIB each of 10 **.**

Group IIA (Vit D treated group):(n=10)

Ten diabetic rats received vitamin D (1,25-dihydroxyvitamin D_3) with a dose of (0.03 µg/kg/day) dissolved in corn oil, administrated orally using a gavage needle. The dose was 1 µg =40 IU and this treatment was continued **for 8 weeks.**[21]

Group IIB (untreated group): (n=10)

These ten diabetic rats received the vehicle oral gavage per day for (8 weeks) .

Both control and diabetic untreated rats received an equivalent administration of corn oil for 8 weeks.[21]

Body Weight of rats was measured at the beginning of the experiment and at week 8 and then after 16 weeks at the end of the experiment.

At the end of the experiment, rats were fasted overnight and measuring the weight plexus of the rat by capillary under light ether anesthesia. [22] The serum was separated by centrifugation at 3000 rpm for 15 minutes and was stored at -20^{0}C until assayed for measuring the following parameters:

1. Fasting blood glucose level by colorimetric method. [19]
2. Serum insulin level by enzyme linked immunosorbent assay (ELISA).[23]
3. Insulin resistance via the (HOMA-IR).[23]
4. Serum Liver enzymes; alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity by colorimetric method.[24]
5. Serum super oxide dismutase (SOD) activity by colorimetric method.[25]
6. Serum IL1β level by ELISA.[26]

Liver tissue preparation

The rats were sacrificed with decapitation, the anterior abdomen was incised to expose the liver. The liver from each rat was excised immediately after perfusion and rinsed with ice-cold saline solution. The ventral median lobe of the liver was fixed in 10% neutral buffered formalin for histopathological study. The remaining portion was homogenized in 0.9% saline, centrifuged at 400×g for 15 minutes and the supernatant was decanted and stored at 70^{0}C until used for 7.Reverse transcription real time quantitative-polymerase chain reaction of (RT-qPCR) was performed on liver tissue to quantify gene expression level of JNK.[27, 28]

Results

Table (I): Comparison between the three studied groups regarding body weight (g) .

Body weight	Group I (n = 10)	Group IIA (n = 10)	Group IIB (n = 10)	Test of sig.	P
At the beginning of the experiment1	**Group I (n = 10)**	**Group II (n = 20)**		t= 0.231	0.819
Min. – Max.	130.0 – 150.0	130.0 – 150.0			
Mean ± SD.	139.7 ± 5.93	139.15 ± 6.23			
Median	140.5	139.0			
At 8 weeks					
Min. – Max.	138.0 – 155.0	202.0 – 339.0	200.0 – 230.0	F= 40.869*	<0.001*
Mean ± SD.	147.5 ± 5.87	268.6[a] ± 50.66	218.2[ab] ± 10.76		
Median	147.0	270.0	220.5		
Sig. bet. grps	$p_1<0.001^*, p_2<0.001^*, p_3=0.002^*$				
At the end of the experiment					
Min. – Max.	165.0 – 238.0	177.0 – 299.0	171.0 – 218.0	F= 10.485*	<0.001*
Mean ± SD.	189.8 ± 20.95	246.5[a] ± 46.32	193.4[b] ± 17.29		
Median	186.5	244.5	191.0		
Sig. bet. grps	$p_1=0.001^*, p_2=0.964, p_3=0.002^*$				

t: Student t-test

F: F for ANOVA test, Pairwise comparison bet. each 2 groups was done using Post Hoc Test (Tukey)

p: p value for comparing between the three studied groups

p_1: p value for comparing between group I and group IIA

p_2: p value for comparing between group I and group IIB

p_3: p value for comparing between group IIA and group IIB

*: Statistically significant at $p \leq 0.05$

GroupI: Control

Group IIA: Vitamin D treated

Group IIB: Untreated

a: significant with Group I

b: significant with Group IIA

Table (II): Comparison between the three studied groups regarding fasting blood glucose levels (mg/dl).

Fasting blood glucose(mg/dl)	Group I (n = 10)	Group IIA (n = 10)	Group IIB (n = 10)	F	P
At 9 weeks					
Min. – Max.	68.0 – 95.0	311.0 – 365.0	310.0 – 370.0		
Mean ± SD.	77.80 ± 9.27	336.80[a] ± 22.49	333.3[a] ± 20.32	658.84*	<0.001*
Median	74.50	341.50	328.0		
Sig. bet. grps	$p_1<0.001^*, p_2<0.001^*, p_3=0.904$				
At the end of the study					
Min. – Max.	63.0 – 97.0	63.0 – 100.0	130.0 – 185.0		
Mean ± SD.	74.80±10.14	86.90±12.80	151.1[ab]±17.93	85.774*	<0.001*
Median	72.0	93.50	148.5		
Sig. bet. grps	$p_1=0.149, p_2<0.001^*, p_3<0.001^*$				
p_4	**0.558**	**<0.001***	**<0.001***		

F: F for ANOVA test, Pairwise comparison bet. each 2 groups was done using Post Hoc Test (Tukey)

p: p value for comparing between the three studied groups

p_1: p value for comparing between group 1 and group 2A

p_2: p value for comparing between group 1 and group 2B

p_3: p value for comparing between group 2A and group 2B

p_4: p value for comparing between the same groups at the 9 weeks and at the end of the experiment

*: Statistically significant at $p \leq 0.05$

Group 1: Control

Group 2A: Vitamin D treated

Group 2B: Untreated

a: significant groups 2A , 2B versus group1

b: significant with Group 2A

Table (III): Comparison between the three studied groups regarding Serum Insulin and HOMA-IR

Sugar picture	Group I (n = 10)	Group IIA (n = 10)	Group IIB (n = 10)	F	p
Serum Insulin (Pg/ml)					
Min. – Max.	1857.0 – 2354.0	2203.0 – 2927.0	2315.0 – 3560.0		
Mean ± SD.	2053.6 ± 181.8	2551.3[a]± 220.1	2900.0[ab]±440.8	19.658*	<0.001*
Median	1973.0	2557.0	3003.0		
Sig. bet. grps	p_1=0.003*,p_2<0.001*,p_3=0.041*				
HOMA-IR					
Min. – Max.	7.98–11.25	8.51–17.04	19.51–33.69		
Mean ± SD.	9.37±1.09	13.66[a]±2.61	26.76[ab]±4.38	90.431*	<0.001*
Median	9.03	14.09	27.64		
Sig. bet. grps	p_1=0.010*,p_2<0.001*,p_3<0.001*				

F: F for ANOVA test, Pairwise comparison bet. each 2 groups was done using Post Hoc Test (Tukey)

p: p value for comparing between the three studied groups

p_1: p value for comparing between group I and group IIA

p_2: p value for comparing between group I and group IIB

p_3: p value for comparing between group IIA and group IIB

*: Statistically significant at $p \leq 0.05$

Group I: Control

Group IIA: Vitamin D treated

Group IIB: Untreated

a: significant with Group I

b: significant with Group IIA

Table (IV): Comparison between the three studied groups regarding serum ALT (U/ml) ,serum AST(U/ml) .

Investigation laboratory	Group I (n = 10)	Group IIA (n = 10)	Group IIB (n = 10)	F	p
Serum ALT (U/ml)					
Min. – Max.	15.0 – 37.0	11.0 – 28.0	22.0 – 28.0		
Mean ± SD.	21.0 ± 8.64	17.60 ± 6.13	25.0[b]± 2.16	3.520*	0.044*
Median	19.0	16.0	24.50		
Sig. bet. grps	p_1=0.453,p_2=0.339,p_3=0.034*				
Serum AST (U/ml)					
Min. – Max.	32.0 – 70.0	32.0 – 49.0	43.0 – 98.0		
Mean ± SD.	45.20 ± 14.32	41.10 ± 7.28	61.70[b]± 20.33	5.315*	0.011*
Median	43.0	43.0	56.0		
Sig. bet. grps	p_1=0.814,p_2=0.051,,p_3=0.013**				

F: F for ANOVA test, Pairwise comparison bet. each 2 groups was done using Post Hoc Test (Tukey)

p: p value for comparing between the three studied groups

p_1: p value for comparing between group I and group IIA

p_2: p value for comparing between group I and group IIB

p_3: p value for comparing between group IIA and group IIB

*: Statistically significant at $p \leq 0.05$

Group IControl

Group IIA:Vitamin D treated

Group IIB:Untreated

a:significant with Group I

b:significant with Group IIA

Table (V): Comparison between the three studied groups regarding Serum SOD (U/ml) and IL-1β Eliza (Pg/ml)

	Group I (n = 10)	Group IIA (n = 10)	Group IIB (n = 10)	F	p
Serum SOD (U/ml)					
Min. – Max.	210.0 – 233.0	152.0 – 273.0	142.0 – 203.0		
Mean ± SD.	222.4 ± 8.40	219.5 ± 38.13	178.4[ab]± 23.51	8.744*	0.001*
Median	223.0	226.5	177.0		
Sig. bet. grps	p_1=0.967,p_2=0.002*,p_3=0.005*				
IL-1β Eliza (Pg/ml)					
Min. – Max.	513.7 – 618.4	410.5 – 844.7	571.8 – 923.4		
Mean ± SD.	563.5 ±41.27	678.9 ± 128.2	761.7[a]± 121.4	9.041*	0.001*
Median	560.0	716.9	804.2		
Sig. bet. grps	p_1=0.052,p_2=0.001*,p_3=0.199				

F: F for ANOVA test, Pairwise comparison bet. each 2 groups was done using Post Hoc Test (Tukey)
p: p value for comparing between the three studied groups
p_1: p value for comparing between group I and group IIA
p_2: p value for comparing between group I and group IIB
p_3: p value for comparing between group IIA and group IIB
*: Statistically significant at $p \leq 0.05$
GroupI: Control
Group IIA: Vitamin D treated
Group IIB: Untreated
a: significant with Group I
b: significant with Group IIA

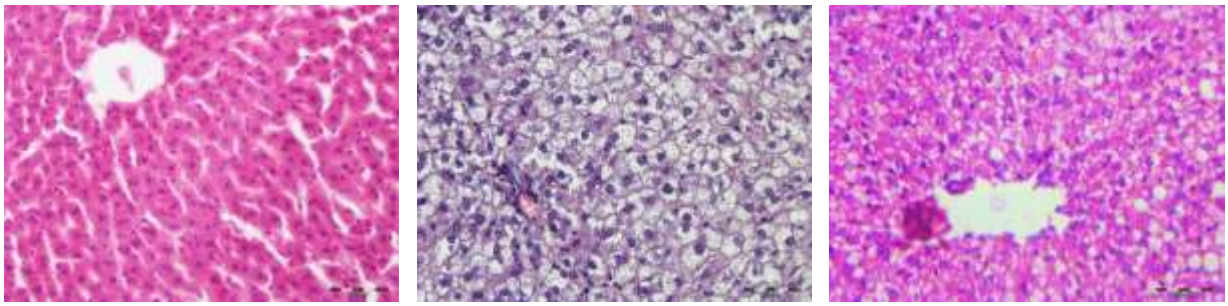
Table (VI): Comparison between the three studied groups regarding ΔΔCT (unit ?)

	Group I (n = 10)	Group IIA (n = 10)	Group IIB (n = 10)	F	P
ΔΔCT(unit ?)					
Min. – Max.	0.02 – 0.10	0.04 – 1.05	1.0 – 1.0		
Mean ± SD.	0.07 ± 0.03	0.25 ± 0.31	1.0[ab]± 0.0	76.407*	<0.001*
Median	0.09	0.12	1.0		
Sig. bet. grps	p_1=0.091,p_2<0.001*,p_3<0.001*				

F: F for ANOVA test, Pairwise comparison bet. each 2 groups was done using Post Hoc Test (Tukey)
p: p value for comparing between the three studied groups
p_1: p value for comparing between group I and group IIA
p_2: p value for comparing between group 1 and group IIB
p_3: p value for comparing between group 2A and group IIB
*: Statistically significant at $p \leq 0.05$
Group I: Control
Group IIA: Vitamin D treated
Group IIB: Untreated
a: significant with Group I
b: significant with Group IIA

Effects of vitamin D on liver histopathology

In the control group, the structure of liver is normal and clear. Various pathological changes appeared in the untreated group, including narrow liver sinusoid, distortion of liver architecture, hepatocyte swelling, fatty degeneration, cytoplasm rarefaction, spotty necrosis scattering in the hepatic lobule as well as inflammatory cell infiltration. However, these changes described above were markedly improved in the VD treated group



Control group Untreated group Vit D treated group

Figure (1): Changes in liver histology (magnification, × 400; stain, hematoxylin and eosin). In the control group, liver cell alignment is normal. In the untreated group, liver cells appear swelled with fatty degeneration and inflammatory cell infiltration. In the vit D treated group, fatty degeneration and inflammatory lesions are alleviated, in comparison with untreated group.

Discussion

It has been demonstrated that inflammation and IR play an initial role in the onset of T2DM and the progression of its complications. Vitamin D is a well-known steroid hormone that has been identified as a regulator of inflammation and IR. Based on available clinical and epidemiological data, the positive effects of vitamin D appear to be primarily related to its action on inflammation and secondary to its action on insulin sensitivity and secretion.

In our study, at the end of the experiment there was significant decrease in body weight in Vit D treated group and untreated group compared by their weight at the 8 weeks. But the BWt was significantly increased in Vit D treated group in relation to untreated group. Most probably the weight loss in untreated group occurred as a result of increased muscle wasting owing to loss of tissue proteins instead of using glucose in aerobic glycolysis. The improvement in BWt in Vit D treated group is suggested to be due to its effect on insulin sensitivity and glucose metabolism.[29, 30] Vitamin D influences β-cell insulin secretion through a rise in intracellular calcium concentration via non-selective voltage-dependent calcium channels. [31, 32]Similar results were found by Rossmeisl et al., 2003. [33]

Moreover, at the end of the experiment, there was a statistically significant decrease in fasting blood glucose level in group IIB (untreated) and group IIA (Vit D treated group) in relation to control, yet still significantly higher in group IIB than group IIA. Blood glucose return to normal in group IIA may be a result of Vit D improved insulin resistance and improved insulin signaling pathway, [34] Benetti et al., 2018 [35] demonstrated that animals treated with vitamin D showed a significant improvement of glucose tolerance.

In the present study, at the end of the experiment, serum insulin in the untreated group was significantly increased in comparison to control and Vit D treated group. This decrease in serum insulin level in Vit D treated group, supports the evidence that the beneficial effects of vitamin D were mediated through the modulation of VDR a receptor of vitamin D thereby improving signal transduction in the treatment of diabetes-induced liver complications.[36, 37] Benetti, et al[35] demonstrated similar results .

In the present work, HOMA-IR in un treated group was significantly increased in relation to control and Vit D treated group. It has been well documented that HFD causes insulin resistance and also reductions in GLUT4 protein levels which are usually associated with increased insulin resistance.[36, 38] Also significant decreased of HOMA-IR in Vit D treated group suggested that Vit D improves insulin resistance; which was also found by Zhuo et al[39]

In the present study, serum ALT was significantly increased in the untreated group compared with Vit D treated group. Serum AST showed the same pattern, due to the role of 1, 25 $(OH)_2D_3$ in regulating pro inflammatory cytokines and lipid metabolism in a diabetic liver model. [40] [41][41][41]Ning et al[42] demonstrated similar results

In present work serum SOD between significantly increased in the Vit D treated group and significantly decreased in untreated group, suggesting that vitamin D played a vital role in the protection of tissues from damage by free radicals as a non-enzymatic antioxidant compound. [43, 44] Claro da Silva Tet al work is supporting our results. [45].

In the present study, there was a significantly increased Serum interleukin 1β in untreated group compared to Vit D treated one and in the control group, supporting the role of 1,25-dihydroxy vitamin D as an anti-inflammatory agent .1,25-(OH)2D3 inhibits the release of the pro-inflammatory cytokine [46, 47] , Similar result shown by Liu et al[20] .

In this study, RT-qPCR analysis showed that the expression level of JNK which are implicated in inflammation and insulin resistance, increased in untreated and decreased following treatment with vitamin D. Based on these results, we speculate that vitamin D exerts therapeutic effects on diabetes-induced liver complications, possibly by downregulating the expression of JNK. This modulation may be in part due to the anti-inflammatory effect of vitamin D; however. the underlying mechanism remains to be elucidated[20]. Our findings are supported by Liu et al. [20]

The histopathological study of liver was performed to assess the direct effects of vitamin D on diabetes-induced liver complications. The results showed that the administration of vitamin D significantly alleviated certain pathological changes, particularly steatosis and inflammatory cell infiltration.

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