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The Potential Role of Vitamin D Supplementation in Ameliorating the Pathogenesis of Induced Type 2 Diabetes in Rats

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ABSTRACT

Background: Vitamin D act as a pre-hormone in a wide range of skeletal and extra-skeletal functions. The therapeutic potentials of vitamin D cover some very critical areas including diabetes mellitus.

Objectives: The study aimed to assess the role of vitamin D supplementation on glycemic control and insulin resistance in rats with induced type 2 diabetes mellitus (T2DM).

Materials and methods: Sixty male Albino Wistar mature rats, weighing 180±10 g and divided into 5 groups. **Normal control group**: 10 rats kept on regular diet and received no treatment. **Normal + 1000IU vitamin D group**: 5 rats kept on regular diet with 1000IU vitamin D supplementation. **Diabetic control group**: 15 rats with streptozotocin (STZ) induced diabetes mellitus and high fat diet receiving no treatment. **Diabetic + 1000IU vitamin D group:** 15 rats with STZ induced diabetes mellitus, fed high fat diet and supplemented with 1000IU vitamin D. **Diabetic + 2000IU vitamin D group**: 15 rats with STZ induced diabetes mellitus, fed high fat diet and supplemented with 2000IU vitamin D.

Results: There was significant reduction of serum glucose, serum insulin and homeostatic model assessment of insulin resistance (HOMA IR), total cholesterol, LDH-c

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and significant increase of HDL-c in Diabetic + 1000IU Vit. D and Diabetic + 2000IU Vit. D groups, when compared to diabetic group. In pancreatic and hepatic tissues, a significant increase of glucose transporter 2 (GLUT-2), insulin receptor (IR), vitamin D receptors (Vit. DR) gene expressions in diabetic + 1000IU Vit. D and diabetic + 2000IU Vit. D groups, when compared to diabetic rats was noticed ($p<0.05$). In muscle tissue, a significant increase of gene expression of GLUT-4 in diabetic + 1000IU Vit. D and diabetic + 2000IU Vit. D groups, when compared to diabetic rats. All results of Diabetic + 2000IU Vit. D was significantly better than of Diabetic + 1000IU Vit. D group. Gel Electrophoresis of the polymerase chain reaction (PCR) showed that diabetes mellitus results in increased methylation of GLUT-2 gene which is partially reversed by treatment with 1000IU and 2000IU vitamin D. The same can be implied from amplified GLUT-2 gene electrophoresis of hepatic tissue samples.

Conclusion: Supplementations with vitamin D increased GLUT-2 gene expression in hepatic and pancreatic tissues of diabetic rats by reducing their methylation which was associated with improvement in glycemic state.

Keywords: *Vitamin D, Type 2 Diabetes, Glycemic Control, GLUT-4*

INTRODUCTION

Vitamin D is a fat-soluble vitamin that mainly functions in calcium homeostasis and bone mineralization. Deficiency of vitamin D, which is considered a pandemic recently, is associated with skeletal manifestations including rickets in early years of life and osteomalacia in older ages. The effects of vitamin D deficiency extend to include wide range of extra-skeletal including cardiovascular, neuropsychiatric, endocrinal, gastrointestinal, and renal effects[(1)].

On the other hand, type 2 diabetes mellitus (T2DM) is one of the most prevalent diseases with very high burden and complication rates. In contrast to type 1 diabetes mellitus, T2DM is mainly associated with impaired sensitivity to the insulin secreted by still functioning β-cells of the pancreas. Increased insulin resistance, despite the extensive studies conducted, is still not fully explained due to the complex relation between insulin receptors (IR), glucose transporters (GLUTs) in different tissues, plasma membrane fluidity, intracellular signaling and transcriptional regulation of metabolism[(2)].

Evidence on association between vitamin D deficiency and T2DM is accumulating over the past decade[(3)]. Vitamin D deficiency shares the same risk

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factors with T2DM including American-African race, obesity, aging and low physical activity[4].

Reports suggested causative relationship between vitamin D deficiency and T2DM based on the findings related to role of vitamin D in glucose homeostasis, insulin secretion and insulin sensitivity[5,6].

There is a growing interest over the potential glucostatic and insulin secretagogue role of vitamin D[4]. However, the exact role of vitamin D in diabetes mellitus especially T2DM is still debatable with conflicting findings from study to study. The exact physiological and molecular mechanisms behind the reported positive findings are also not fully elucidated.

So, this study aimed to assess the potential role of vitamin D as a nutritional supplement in T2DM-rat model induced by streptozotocin injection, to examine the exact effect on glycemic control and insulin resistance and to highlight the underlying pathophysiological mechanisms.

MATERIALS AND METHODS

Sixty male Albino Wistar rats supplied from The Egyptian Organization for Biological products and Vaccines (Cairo, Egypt), were used. The rats were of mature, weighing 180±10 g at start of experiment with normal vital signs and base line laboratory tests. The rats were housed in stainless steel cages under conventional housing conditions for 10 days to acclimatize to the new environment before start of the experiments with room temperature around 25°C and 12 hours light/dark cycles. At this stage rats were fed rodent chow diet (El-Nasr Pharmaceuticals and Chemicals Industry, Egypt) with free access to drinking water. This study was approved by ethics committee of the Faculty of Pharmacy, Mansura University, Mansura 35516, Egypt, that complies with "Principles of Laboratory Animal Care" (National Institute of Health publication No. 85-23, revised 1985).

Animals were divided into five groups:

- **Normal control group**: 10 rats kept on regular diet and received no treatment.
- **Normal + 1000IU vitamin D group**: 5 rats kept on regular diet with 1000IU vitamin D supplementation for 45 days.

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- **Diabetic group**: 15 rats with STZ induced diabetes mellitus and high fat diet receiving no treatment for 45 days.
- **Diabetic + 1000IU vitamin D group**: 15 rats with STZ induced diabetes mellitus, fed high fat diet and supplemented with 1000IU vitamin D for 45 days.
- **Diabetic + 2000IU vitamin D group**: 15 rats with STZ induced diabetes mellitus, fed high fat diet and supplemented with 2000IU vitamin D for 45 days.

Induction of diabetes mellitus:

Experimental diabetes was induced by feeding rats with high-fat diet for 8 weeks followed by a single intra-peritoneal injection of 60 mg/kg of streptozotocin (STZ), freshly dissolved in cold 0.1 M citrate buffer, pH 4.5 after 15 min of intra-peritoneal injection of nicotinamide (110 mg/kg) prepared in normal saline. Control rats received standard diet and were injected with citrate buffer alone[(7)]**.**

Administration of vitamin D:

The vitamin D3-treated groups received cholecalciferol (Vidrop oral drops, Novartis Co, Switzerland) via gavage once daily in the doses of 1000 IU or 2000 IU [(8)] for a period of 45 days.

Blood and tissue sampling:

Blood samples were collected at the end of the study. Animals were then decapitated and tissue samples from liver, pancreas and muscles were collected. Tissue samples were frozen in -80^{o}C for further polymerase chain reaction (PCR) assessment of gene expression.

Biochemical analysis:

Estimation of blood glucose, total cholesterol, triacyleglycrol, high density lipoprotein-cholesterol (HDL-c) by colorimetric methods was done. Low density lipoprotein-cholesterol (LDL-c) was calculated using Friedwald Formula [(9)]**..** Serum insulin was estimated by ELISA method.

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Molecular analysis:

Analysis of tissue expression of insulin receptor (IR), Glucose transporter-2 (GLUT-2), glucose transporter -4 (GLUT-4), and vitamin D3 receptor (VDR) was done by quantitative real time PCR (RT-PCR) amplification.

Real time PCR analysis:

Total RNA isolation was done using the easy-spin^{TM} total RNA extraction kit (iNtron Biotechnology, South Korea). Reveres transcription was done using HiSenScript^{TM} RH (-) cDNA synthesis Kit (iNtron Biotechnology, South Korea). Master mix Solution (i-Taq^{TM}) (iNtron Biotechnology, South Korea – Ref: 25027) was used for quantitative amplification. The assay was run on Step One™ Real-Time PCR System version 3.1 (Applied Biosystems, USA) according to the manufacturer's instructions. Primers sequences are shown in table 1. The amplitude of change of the expression observed was analyzed by the $2^{-\Delta\Delta CT}$ method.

Methylation-specific PCR (MSP) analysis for GLUT2 gene:

DNA was extracted from blood and from tissue by DNA isolation kits (QIAamp DNA Minikit, QIAGEN GmbH, Hilden, Germany) according to the instructions of manufacturer. Then DNA was treated with sodium bisulfite with a commercial kit (EpiTect Bisulfite, QIAGEN) following the manufacturer's protocol. Methylation-specific PCR (MSP) analysis was done for GLUT-2 according to **Rajagopal et al. [(10)]**. The primers sequence used for methylation analysis was shown in **table 1**. The products of PCR were loaded into a 3% ethidium bromide stained agarose gel and visualized under UV illumination.

Statistical analysis:

Data was computerized and statistically analyzed using SPSS version 25. Quantitative parametric data was expressed as mean ± standard deviation and compared with ANOVA test. Quantitative non parametric data was expressed as median and range and compared with kruskal-wallis test. Post hok tukey test was used for comparison between each two groups. P value < 0.05 was considered to be significant and < 0.001 highly significant.

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RESULTS

Blood biochemistry:

There were no significant differences between normal + 1000IU Vit. D group and normal control group. There were significant elevation of serum glucose, insulin, HOMA IR, total cholesterol and LDL-c, while there was a significant decrease of HDL-c in diabetic group when compared to normal control group. On the other hand, there were significant reduction of serum glucose, insulin, HOMA IR, total cholesterol and LDH-c, with a significant increase of HDL-c in both Diabetic + 1000IU Vit. D and Diabetic + 2000IU Vit. D groups when compared to diabetic group. The results of Diabetic + 2000IU Vit. D is better than that of Diabetic + 1000IU Vit. D (**Table 2**).

Results of gene expression analysis:

In pancreatic and hepatic tissues, there were a significant reduction of GLUT-2, IR, and VDR gene expression in diabetic rats when compared to normal rats with 1000 IU Vit. D. A significant increase of GLUT-2, IR, VDR gene expression in both diabetic + 1000IU Vit. D and diabetic + 2000IU Vit. D groups when compared to diabetic rats were noticed ($p<0.05$). Moreover, there were a significant increase of GLUT-2, IR, and VDR gene expression in diabetic rats treated with 2000 IU Vit. D when compared to diabetic rats treated with 1000 IU Vit. D (**Table 3**).

In muscle tissue, there was a significant decrease of gene expression of GLUT-4 in diabetic rats when compared to normal + 1000 IU Vit. D group, while there was a significant increase in both diabetic + 1000IU Vit. D and diabetic + 2000IU Vit. D groups when compared to diabetic rats. Also, a significant increase of GLUT-4 in diabetic + 2000IU Vit. D group when compared to diabetic + 1000IU Vit D group was showed ($p<0.05$) (**Table 3**).

Gel electrophoresis of the PCR yield of both the methylated and un-methylated GLUT-2 gene in the pancreatic tissue samples shows that diabetes mellitus results in increased methylation of GLUT-2 gene which is partially reversed by treatment with 1000 IU vitamin D as well as 2000 IU vitamin D. The same can be

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implied from amplified GLUT-2 gene electrophoresis of hepatic tissue samples (**Figure 1**).

DISCUSSION

Vitamin D role as a pre-hormone is extending to a wide range of skeletal and extra-skeletal functions. The research addressing therapeutic potentials of vitamin D is going feverish and covers some very critical areas including diabetes mellitus. In this experimental case control study, we aimed at addressing the potential role of vitamin D administration to diabetic rats and to study the underlying molecular mechanisms.

In this study we confirmed that vitamin D administration improves the glycemic control and reduces insulin resistance in Wister rats with T2DM. Furthermore, Vitamin D improves the serum cholesterol profile of diabetic rats fed high fat diet. The improvement in serum glucose and insulin levels as well as insulin resistance assessed by HOMA-IR after administration of vitamin D has been confirmed. **De Souza Santos and Vianna**[(11)] showed that cholecalciferol can successfully lower blood glucose level in experimental models of diabetes mellitus. Similar findings were reported in rats with high fat diet induced metabolic syndrome[(12)]. Vitamin D receptor antagonists were proved to have favorable effects on T2DM Sprague Dawley rats[(13)]. Chronic oral supplementation of diabetic obese rats with vitamin D (for 70-80 days) has led to significant decrease in insulin secretion and reduction of insulin resistance[(14)]. **Elseweidy et al.**[(15)], showed that vitamin D effectively lowers insulin resistance and contributes to glycemic control albeit not providing the exact duration of vitamin D treatment.

Some studies showed that vitamin D increase insulin secretion which could be due to the presence of vitamin D response element (VDRE) in the insulin gene promoter area of β islets[(16)]. **Derakhshanian et al.**[(17)], showed that vitamin D increases insulin like growth factor 1 (IGF-1) and insulin levels in experimental rats. Also, vitamin D was found to potentiate glucose stimulated insulin secretion in culture β islets of pancreas of rats and human [(18)]. In these regards it is more reasonable to point to vitamin D as an insulinstat other than just stimulator or inhibitor of insulin secretion.

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The current study shows that vitamin D improves the cholesterol profile but not triacylglycrol which may be explained by the concomitant supplementation of high fat diet. **Elseweidy et al.**[15], showed similar effects of vitamin D on cholesterol profile which was associated with significant lowering of triacylglycrol. In the study by **Mostafa et al.**[12], lowering of total cholesterol, LDL-c and triacylglycrol as well as elevation of HDL-c was reported. In this study, caloric stress and STZ were used for induction of diabetes mellitus.

The mechanisms behind glucostatic functions of vitamin D may be too complex to be simply explored in a single study. The current study aimed at highlighting the main features of vitamin D action to open the doors for more in-depth future investigations. The targets for investigation were glucose and insulin receptors in the main tissues involved in glucostasis, pancreas, liver and muscles. Pancreatic β islets cells are known to express both GLUT-1 and GLUT-2 in an opposite direction with the former being more expressed at low glucose concentrations and the latter being more effectively induced by high glucose concentrations[19]. The GLUT-2 receptor is the main receptor regulating glucose uptake in pancreas and liver which is not the case in muscle tissues where GLUT-4 is transporter.

The current study provided the evidence that pancreatic tissues of diabetic rats show lower expression of GLUT-2 and IR compared to tissues from normal rats. Vitamin D restored the expression of these two receptors to normal levels in a dose dependent fashion. The role of GLUT-2 in insulin secretion and its relation to diabetes has been a topic of extensive discussion[20–23]. Despite, the relation between vitamin D deficiency and diabetes has been deeply investigated; the relation of vitamin D to pancreatic expression of GLUT-2 and IR is yet uncovered. In an experimental study, absence of GLUT-2 in rats with static magnetic field induced diabetes was corrected by vitamin D administration[24]. Induction of diabetes by static magnetic field is not well addressed and the down regulation of GLUT-2 expression may be just transient and cannot be directly attributed to vitamin D. The paucity of studies investigating this direct relation allows us to explicitly describe it as a unique finding that should be further investigated.

Vitamin D supplementation has similar effects on insulin-responsive tissues. In the current study vitamin D at 2000IU dose could effectively increase GLUT-2 and

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IR expression in hepatic tissues of diabetic rats. Similar effects on IR were reported by **Maestro et al.**[25] and **George et al.**[26]**,** which was not the case in some other studies[27,28]. In a systematic review on STZ-induced diabetic rats, vitamin D supplementation has led to significant elevation in IR expression[29]. Vitamin D deficient rats were shown to have lower hepatic expression of GLUT-2[30]. **George et al.**[26], showed that supplementation of vitamin D to diabetic rats significantly increased hepatic expression of GLUT-2. The lack of significance of 1000IU vitamin D effect may be due to the small sample size not statistically powered enough yet it is still clear the effect is dose dependent.

We have showed that muscles of diabetic rats have lower expression of GLUT-4 and higher expression of IR compared to normal rats which was partially reversed after vitamin D administration in a dose dependent manner. It is well known that GLUT-4 is the key regulator of glucose uptake by insulin sensitive tissues including muscles and adipocytes and was shown to be down regulated in diabetic models[28,31,32]**.** It has been suggested by some studies that the ability of vitamin D to improve glucose homeostasis is through the regulation of GLUT-4 expression in insulin sensitive tissues. **Tamilselvan et al.**[33], showed that vitamin D improves GLUT-4 expression in cultured L6 myotubes. **Alkharfy et al.**[28], failed to translate the *in vitro* effects of vitamin D on expression of GLUT-4 into *in vivo* findings when they used experimental mice models of diabetes. While they did not provide logical explanation, the dose at which vitamin D was administered may be behind the inconsistent results. In another study, the effects vitamin D treatment to diabetic women extended beyond molecular changes to improve motor neuropathy and reflexes [34].

The relation of diabetes mellitus to IR expression in muscle tissue is less well elucidated. In our study IR was found to be up regulated in diabetic rats, which was not usually the case in all the other study. **Alkharfy et al.**[28], showed similar relation of diabetes to IR expression in muscle tissues with similar effects of vitamin D. On the contrary, **Xavier et al.**[32], showed that diabetic rats' muscles express lower level of IR which was also reversed by vitamin D. A possible explanation can be the method of diabetes induction. **Alkharfy**[28], and his colleagues used high fat diet, while **Xavier**[32], and his colleagues used STZ only. The high fat diet is known to affect insulin sensitivity and hence may induce expression of IR in return.

Finally, in attempt to understand the mechanisms behind our findings, we performed analysis of vitamin D receptor (VDR) expression in all and degree of methylation of GLUT-2 gene. The VDR expression was found to decrease in diabetic rats and increase after administration of vitamin D. It is obvious that, within the range used, vitamin D actions are enforced by more expression of its receptor which may explain the dose dependent effects of vitamin D which is supported by the very recent study by **Morró et al.**[(35)]. Studies have shown that diabetes mellitus affects the degree of GLUT-2 gene methylation[(36)]. Vitamin D is known to affect methylation of wide range of genes [(37)].

In conclusion vitamin D supplementation increased GLUT-2 gene expression in hepatic and pancreatic tissues of diabetic rats by reducing their methylation which was associated with improvement in glycemic state. Our finding in this regard, despite unique, will need further investigation to confirm that this change in methylation is the direct cause of change in GLUT-2 gene expression or amelioration of diabetes.

Abbreviations:
GLUT-2: Glucose transporter-2; HDL-c: High density lipoprotein-cholesterol; HOMA-IR: Homeostatic model assessment of insulin resistance; IR: Insulin receptor; LDL-c: Low density lipoprotein-cholesterol; VDR: Vitamin D receptor.

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Table 1: Primers sequence for all studied genes

Gene	Primers sequence
GAPDH (Reference gene)	F: 5′ GGCACAGTCAAGGCTGAGAATG 3′ R: 5′ ATGGTGGTGAAGACGCCAGTA 3′
IR	F: 5′ GCCATCCCGAAAGCGAAGATC 3′ R: 5′ TCTGGGGAGTCCTGATTGCAT 3′
GLUT-4	F: 5′ AGTTGGAAAGAGAGCGTCCACTGT 3′ R: 5′ GCTGCAGCACCACTGCAATAATCA 3′
GLUT-2	F: 5′ GTCAGAAGACAAGATCACCGGA 3′ R: 5′ AGGTGCATTGATCACACCGA 3′
VDR	F: 5′ GATGCTGGTGCTGAGTATGTCG 3′ R: 5′ GTGGTGCAGGATGCATTGCTCTGA 3′
Methylated GLUT-2	F: 5′ ATTAAGOGGTATTGAGGAcGTTA 3′ R: 5′ TTAAGGGGTATTGAGGATGTTA′
Un-methylated GLUT-2	F: 5′ CAATACCACCACAATAAACTACCG′ R: 5′ TACCACCACAATAAACTACCAATAATTC′

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; IR: Insulin receptor; GLUT-2: Glucose transporter-2; GLUT-4: Glucose transporter-4; VDR: Vitamin D receptor.

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Table (2): Blood chemistry results at the end of the study

Test		Normal control	Normal + 1000IU Vit. D	Diabetic	Diabetic + 1000IU Vit. D	Diabetic + 2000IU Vit. D
Glucose homeostasis	Glucose (mg/dl)	85.42 ± 8.11	109.77 ± 8.86	664.50 ± 41.68*	444.02 ± 52.14*†	370.73 ± 48.91*†‡
	Insulin UIU/dl	3.28 ± 0.76	2.70 ± 0.49	15.47 ± 3.15*	8 ± 1.59*†	6.23 ± 1.65*†‡
	HOMA IR	0.70 ± 0.23	0.74 ± 0.18	25.38 ± 5.37*	8.71 ± 1.85*†	5.64 ± 1.38*†‡
Lipid profile	TAG (mg/dl)	149.14 ± 68.80	170.77 ± 14.14	178.87 ± 30.38	179.30 ± 18.11	182.13 ± 23.10
	Total Cholesterol (mg/dl)	105.68 ± 8.43	107.73 ± 2.09	141.56 ± 6.65*	125.67 ± 6.98†	124.10 ± 11.29†
	LDL-c (mg/dl)	38.25 ± 10.60	35.67 ± 3.94	76.08 ± 6.61*	53.23 ± 4.41†	56.91 ± 5.83†
	HDL-c (mg/dl)	37.6 ± 4.72	37.90 ± 1.82	29.70 ± 1.30*	36.57 ± 3.77†	30.76 ± 4.46

Values are represented as (mean ± SD).

HDL-c; high density lipoprotein-cholesterol, HOMA-IR; homeostatic model assessment of insulin resistance, LDL-c; low density lipoprotein-cholesterol and TAG; triacylglycerol.

* P < 0.05 compared to the normal control.
† P < 0.05 compared to the diabetic control.
‡ P < 0.05 compared to the diabetic + 1000IU vit. D.

Table (3): Fold change gene expression in study groups

	Target	Normal + 1000IU Vit. D	Diabetic control	Diabetic + 1000IU Vit. D	Diabetic + 2000IU Vit. D
Pancreas	GLUT-2	1.13 (1.042 – 1.366)	0.016* (0.001 – 0.047)	0.17*† (0.11 – 0.28)	0.45*†‡ (0.22 – 0.56)
	IR	0.98 (0.85 – 1.06)	0.10* (0.07 – 0.17)	0.20*† (0.12 – 0.22)	0.27*†‡ (0.22 – 0.30)
	Vit D R	1.52 (1.19 – 3.34)	0.010* (0.006 – 0.016)	0.10*† (0.08 – 0.12)	0.15*†‡ (0.13 – 0.28)
Liver	GLUT-2	1.006 (0.95 – 1.041)	0.05* (0.03- 0.06)	0.17* (0.04 – 0.43)	0.35*† (0.26 – 0.51)

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	INS R	1.003 (0.766 – 1.361)	0.02 (0.01 – 0.20)	0.18* (0.04 – 0.27)	0.26*† (0.14 – 0.31)
	Vit D R	0.92 (0.82- 1.18)	0.012* (0.003- 0.034)	0.85* (0.01- 0.94)	1.08*† (0.74- 1.21)
Muscle	GLUT-4	1.27 (1.17-1.70)	0.12* (0.08 – 0.30)	0.40*† (0.33 – 0.55)	0.62*†‡ (0.49 – 0.68)

Results are represented as median (range).
GLUT-2; glucose transporter-2, GLUT-4; glucose transporter-4, IR; insulin receptor and VDR; vitamin D receptor.
* P < 0.05 compared to the normal control.
† P < 0.05 compared to the diabetic control.
‡ P < 0.05 compared to the diabetic + 1000IU vit. D.

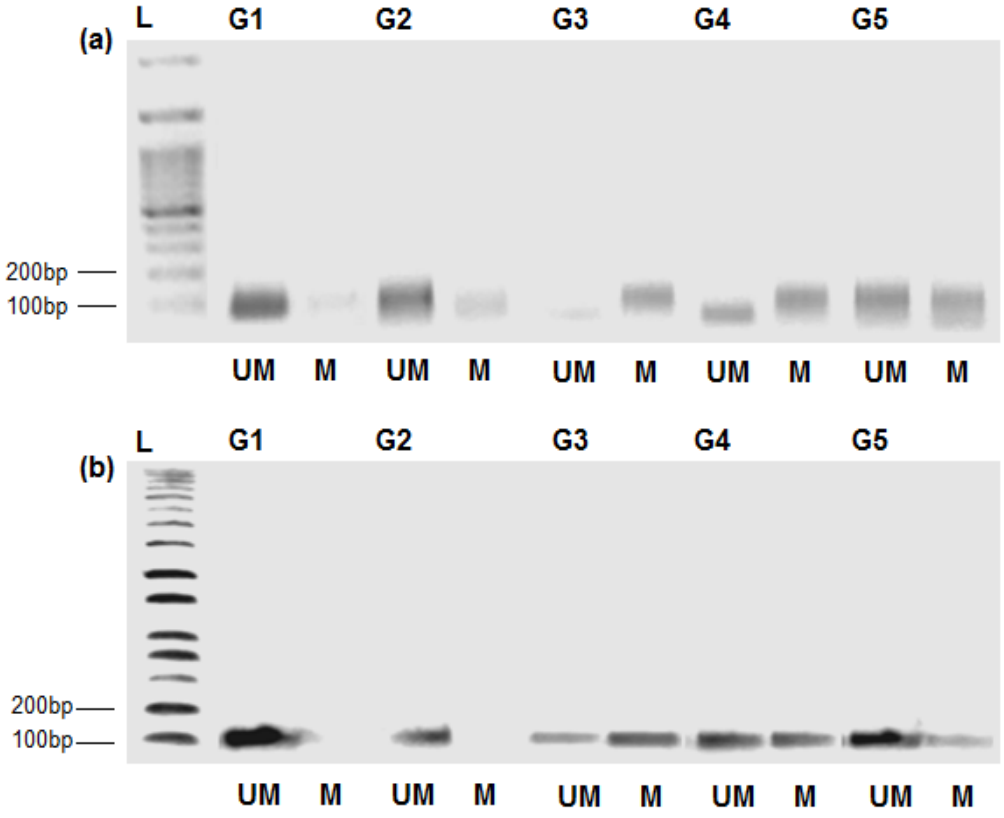

Figure (1): Gel electrophoresis of amplified GLUT-2 gene in pancreas (a) and liver (b).
G1: normal control, G2: normal + with 1000IU Vit. D, G3: diabetic, G4: diabetic + 1000IU Vit. D, G5: diabetic + 2000IU Vit. D and L: 100bp DNA ladder.