Effect of 1, 25 Dihydroxy Vitamin D₃ On Type 2 Diabetes Mellitus
1 Glucose Homeostasis

1.1 Fasting blood glucose levels

Hyperglycemia or increased circulating levels of fasting blood glucose, is one of the cardinal abnormalities of individuals suffering from type 2 diabetes mellitus (T2DM), however hyperglycemia itself can cause insulin resistance. Since elevated glucose levels precedes the onset of this syndrome, FBG levels, is generally used as a diagnostic feature for classifying people who have impaired glucose tolerance (IGT) or diabetes.

<table>
<thead>
<tr>
<th>Normoglycemia</th>
<th>≤ 100 mg/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Impaired fasting glucose (IFG)</td>
<td>100-125 mg/dl</td>
</tr>
<tr>
<td>Impaired glucose tolerance (IGT)</td>
<td>140-199 mg/dl</td>
</tr>
<tr>
<td>Diabetes</td>
<td>≥126 mg/dl</td>
</tr>
</tbody>
</table>

Table 2. Criteria for the diagnosis of diabetes

In this study, the effect of 1, 25 dihydroxyvitamin D3 was observed on the increased fasting blood glucose levels of mice made diabetic by alloxan administration. Figure 2 shows the FBG to be increased in the group with diabetes (250±0.41 mg/dl) marking the proper establishment of diabetic state as compared to the control group 160.97±0.21 mg/dl. The group with diabetes, given 1, 25 dihydroxy vitamin D3 supplementation shows a significant decrease in the FBG levels 190±0.37mg/dl. thus suggesting its role in pathogenesis of hyperglycemia.

1.2 Serum calcium concentration

Phyiological levels of calcium is essential for proper insulin secretion and sensitivity (Bjorklund, A. Lansner, and V. E. Grill 2000), sustained elevations of intracellular calcium may inhibit insulin secretion and the ability of insulin-target cells from sensing the brisk intracellular calcium fluxes necessary for insulin action, such as glucose transport (D. S. Worrall and J. M. Olefsky 2002) resulting in peripheral insulin resistance and thus increased plasma blood glucose levels. Therefore, maintaining the required intracellular calcium levels is of prime importance for proper insulin secretion and sensitivity.
Fig 2.1 depicts the difference in the serum calcium levels in control, diabetic and diabetic+ vitamin D treated groups of mice. The increase in the serum calcium level in the vitamin D treated group 9.5±0.40 mg/ml, in comparison to the decreased levels in diabetic group 7±0.43 mg/ml, is symptomatic of function of 1,25 dihydroxy vitamin D3 in maintaining calcium homeostasis.

1.3 Activity of glucokinase or hexokinase IV

Glucokinase is expressed at highest level in pancreatic β cells and liver. This enzyme catalyzes the transfer of phosphate group from adenosine tri-phosphate (ATP) to glucose, generating glucose-6-phosphate. This step is the first rate-limiting step of glycolysis and thus this enzyme is of prime importance. In liver, this enzyme plays a key role in storing glucose in the form of glycogen and thus controlling the level of glucose in circulation. Therefore, the increase in blood glucose concentration in individuals suffering from T2DM is due to reduced glucose induced insulin secretion from pancreas and reduced glycogen storage into liver.

In this study, it was found that in liver, the activity levels of glucokinase (fig 2.2) decreased in the group with diabetes 0.241±0.014µg/mg/ml as compared to control group 0.75±0.02 µg/mg/ml which was almost restored 0.717±0.003µg/mg/ml on 1,25 dihydroxy vitamin D3 supplementation.

1.4 Activity of glucose 6 phosphatase (G6Pase) and fructose 1,6 bisphosphatase (FBPase)

G6Pase is an enzyme specifically found in liver and kidney that catalyzes the conversion of glucose-6-phosphate to glucose depending upon the concentration of glucose in circulation especially in between meals and extended fasts. Therefore, activity levels of G6Pase can be indirectly related to the presence of sufficient glucose in circulation. The activity of this enzyme can also provide an idea about the rate of gluconeogenesis.

\[
\text{Glucose 6-phosphate} + \text{H}_2\text{O} \rightarrow \text{glucose} + \text{Pi} \quad \Delta G^0 = -13.8 \text{ kJ/mol}
\]

In this study, the activity level of enzyme (fig 2.3) in control group was found to be 0.533±0.02µg/mg/ml however, in diabetic group the level was increased to
0.918±0.008ug/mg/ml, showing a rapid enhancement of gluconeogenesis. On vitamin D supplementation G6Pase activity was found to be significantly decreased to 0.71±0.01ug/mg/ml.

FBPase-1 is an Mg$^{2+}$ dependent enzyme involved in gluconeogenesis which promotes the essentially irreversible hydrolysis of the C-1 phosphate (not phosphoryl group transfer to ADP) thus converting fructose 1, 6 phosphate to fructose-6-phosphate:

\[
\text{Fructose 1,6-bisphosphate} + \text{H}_2\text{O} \rightarrow \text{fructose 6-phosphate} + \text{Pi} \quad \Delta G^0 = -16.3 \text{kJ/mol}
\]

By determining the activity of this enzyme (fig 2.4) we can also indirectly understand the rate of gluconeogenesis and thus know the effectiveness of the treatment given. In our study, the FBPase levels in the control group and diabetic group are found to be 0.10707±0.002ug/mg/ml and 0.433±0.004ug/mg/ml respectively, whereas on 1.25 dihydroxy vitamin D$_3$ supplementation the activity levels were decreased to 0.2733±0.008ug/mg/ml as compared to the diabetic group without supplementation.

2 Effect on oxidative stress

Oxidative stress is a major player in all the chronic illnesses including type 2 diabetes mellitus. Once generated, the free radicals cause pancreatic β cell damage, adding to increased insulin resistance. In addition ROS causes mitochondrial dysfunction lipid peroxidation, glucose oxidation and DNA damage thereby manifesting changes in the histology of diabetic tissues. Oxidative stress thus indirectly cause glucose elevation in circulation, causing hyperglycemia, the main cause of T2DM. There are several parameters analyzed (activity of catalase, superoxide dismutase etc.) in order to demonstrate the effect of 1,25 dihydroxyvitamin D$_3$ on oxidative stress but no significant changes were observed after the treatment. However, a reduction in MDA (malondialdehyde) levels, post treatment was observed. This could be due to the fact that 1,25 dihydroxyvitamin D$_3$ reduces oxidative stress indirectly by normalizing hyperglycemia, which can cause oxidative stress if kept unchecked.

Due to hyperglycemia, more glucose enters the TCA cycle generating an increased concentration of reducing equivalents (NADH and FADH$_2$). This increase pushes...
these electron donors more and more into the ETC, increasing the transfer of protons outside thereby leading to enhanced voltage gradient across the mitochondrial membrane. This rise in voltage gradient when reaches the critical threshold, blocks the further transfer of electrons to complex III. The electrons thereby return to coenzyme Q, which transfers one electron at a time to water, thereby generating superoxide radicals (Peter Proctor, 1989). These radicals once formed increase the production of lipid peroxides by reacting with membrane lipids. Therefore by reducing hyperglycemia, 1,25 dihydroxyvitamin D$_3$ reduces the extent of lipid peroxidation and hence lower MDA levels.

2.1 MDA (malondialdehyde) level estimation

In case of lipid peroxidation, MDA levels were found to be much higher in diabetic mice 0.76±0.03 nmol/mg protein as compared to control group 0.55 nmol/mg protein, although 1, 25 dihydroxyvitamin D$_3$ supplementation decreased the MDA levels 0.644±0.0189 nmol/mg protein significantly (fig 2.5).

3 Estimation of liver function markers (LFT) in serum

In case of type 2 diabetes, there occurs a cardinal abnormality of insulin resistance in peripheral tissues including muscles and adipocytes. This defect in insulin sensitivity leads to increased lipolysis and thus higher levels of circulating non-esterified free fatty acids (NEFA's). Once in circulation these products compete with glucose for oxidation in liver causing a sequential inhibition of pyruvate dehydrogenase, phosphofructokinase and hexokinase II activity besides causing accumulation of diacylglycerol, ceramides and lipids.

These metabolites once accumulated activate phosphorylation of insulin receptor substrate 1 and 2 (IRS-1 and IRS-2) via ser/threo kinase cascade, finally bringing down the downstream events of insulin signalling pathway and contributing further to insulin resistance.

Another mechanism by which type 2 diabetes leads to liver damage is mediated by increased oxidative stress. There occurs an increase in mitochondrial oxidative stress due to excess intracellular fatty acid deposition thereby leading to increased free
radical production which ultimately induces inflammation, cellular necrosis and also fibrosis, all contributing to non alcoholic steohepatitis (NASH).

3.1 **Glutamate pyruvate transaminase (GPT) or alanine transaminase (ALT)**

The increased level of this enzyme in serum is a marker of liver disease. The estimation of the levels of GPT can be related to the extent of diabetes induced liver damage and also to the effectiveness of the treatment given. In control group (fig 2.6) the level of the enzyme was 67±0.34 U/L however, in case of diabetes the levels were increased significantly to 171.40±0.42 U/L. In 1, 25 dihydroxyvitamin D₃ supplemented group there was a marked reduction 80±0.254 U/L in the enzyme level, thus supporting the role of 1, 25 dihydroxyvitamin D₃ in decreasing diabetic complications.

3.2 **Glutamate oxaloacetate transaminase (GOT) or aspartate transaminase (AST)**

GOT (AST) catalyses the transfer of amino group from aspartic acid to 2-oxoglutarate to form and L-glutamate. The oxaloacetate thus forms reacts with 2, 4 dinitrophenylhydrazine to be converted to the corresponding hydrazone which is coloured and thus can be estimated. Serum aspartate transaminase is another disease marker enzyme of liver and its levels were found to be increased in case of diabetic group 167.39±0.45 U/L, as compared to control group 86.52±0.39 U/L however, in 1, 25 dihydroxyvitamin D₃ supplemented group the level was estimated to be 95.64±0.28 U/L, which were much lower than the diabetic group (fig 2.6).

3.3 **Alkaline phosphatase (ALP)**

Alkaline phosphatase is a hydrolase enzyme responsible for removing phosphate groups from many molecules including nucleotides, proteins and alkaloids. High serum levels of ALP are found in diseased conditions. ALP levels were found to be 12.124±0.25 U/L Units in diabetics, 10.694 ±0.37 U/L in control group and 11.12±0.28 U/L in 1, 25 dihydroxyvitamin D₃ supplemented group (fig 2.6).
4 Estimation of renal function markers (RFT) in serum

4.1 Creatinine

The serum creatinine levels in control group was found to be 0.0361± 0.024 mg/100ml which was increased in case of diabetic group to 0.043±0.026 mg/100ml, however on vitamin D supplementation the creatinine levels were reduced to 0.038±0.033 mg/100ml and became closer to the control group (fig 2.7).

4.2 Blood urea and BUN (Blood urea nitrogen)

The levels of blood urea and BUN (fig 2.8) in control group were estimated to be 18.71± 0.41 mg/dl and 35.759±0.29 mg/dl whereas in case of diabetic group it was 22.8±0.17 mg/dl and 48.652±0.36 mg/dl both these parameters depicted a significant increase, however on supplementation with vitamin D the levels were almost normalised to 20.8±0.32 mg/dl and 31.654±0.44 mg/dl. Increase in urea is indicative of negative nitrogen balance which is an index of chronic illness and muscle wasting.

5 Effect on DNA damage

The DNA damage in the tissues of control and treated groups was estimated (fig 2.8, 2.9) using alkaline comet assay. Diabetes caused major DNA damage in liver and pancreas as evidenced by elongated tail length in diabetic group 30.41 ± 2.50μm and 32.45± 2.87 μm respectively when compared to liver and pancreatic cells of control group 7.65 ± 2.20 μm and 6.66 ± 1.20 μm. However, vitamin D supplemented group showed a significant decrease in liver and pancreatic cells DNA tail length with values of 21.80 ± 2.40 μm and 19.25 ± 1.90 μm respectively, indicating a role of VitD3 in controlling the damage caused by persistent hyperglycemia (table 2.1).

6 Histopathological analysis

The histopathological examination of different organs (fig 2.10, 2.11, 2.12) revealed the accumulation of lipid droplets, increased fibrous content, destruction of bile duct and hepatocytic degeneration in liver samples in diabetic group, while in case of kidneys, there was a significant thickening of the glomerular basement membrane, oedema of proximal convulated tubule, hyaline deposition, degeneration of distal
convulated tubule and tubule-interstitial inflammation. We also observed an increase in islet cell population and presence of insulitis in the pancreas of the diabetic group whereas in Vit.D3 supplemented group, there was a significant decrease in all these pathological conditions in liver, kidney and pancreatic tissues (table 2.1).

7 GLUT-4 expression study

The GLUT-4 expression levels were assayed by sandwich elisa in adipocytes and skeletal muscles. It has demonstrated the levels to be 5.65±0.02 ng/ml in adipocytes; 5.122±0.033 ng/ml in skeletal muscles in diabetic group as compared to adipocytes 7.643±0.036 ng/ml; skeletal muscles 9.02±0.039 ng/ml in control group. However, the GLUT-4 levels were significantly improved in Vit.D3 supplemented group; adipocytes 6.9±0.021 ng/ml; skeletal muscles 9.0±0.034 ng/ml. Thus suggesting a role of Vit.D3 in normalizing the GLUT4 expression on the cells. (fig 2.13).

Fasting blood glucose levels in control, diabetic and vitamin D treatment groups

![Graph showing fasting blood glucose levels in control, diabetic, and diabetic + vitamin D treated groups.](image)

Fig. 2 The increase in fasting blood glucose level in diabetic group which decreases on 1, 25 dihydroxy vitamin D3 supplementation
Serum calcium levels in control, diabetic and vitamin D treatment groups

Fig 2.1 The increase in the serum calcium concentration in the diabetic mice with no supplementation; however it became close to the control value in the diabetic group given 1,25 dihydroxy vitamin D$_3$. 
Hexokinase activity levels in control, diabetic and vitamin D treatment groups

Fig 2.2 The decrease in hexokinase level in diabetic group which increases on 1, 25 dihydroxy vitamin D₃ supplementation.
G6Pase and FBPase activity levels in control, diabetic and vitamin D treatment groups

Fig 2.3 The changes in G6Pase and FBPase levels in control, diabetic and 1, 25 dihydroxy vitamin D₃ supplemented group.
Fig 2.4 The extent of lipid peroxidation (MDA levels) in control, diabetic and 1,25 dihydroxyvitamin D$_3$ supplemented group.
SGOT, SGOT and ALP levels in control, diabetic and vitamin D treatment groups

Fig 2.5 The extent enzyme activities of SGOT, SGPT and alkaline phosphatase in control, diabetic and 1,25 dihydroxyvitamin D₃ supplemented group.
Serum creatinine levels in control, diabetic and vitamin D treatment groups

Fig 2.6 The increase in serum creatinine level in diabetic group which decreases on 1, 25 dihydroxy vitamin D₃ supplementation.
Blood urea and BUN levels in control, diabetic and vitamin D treatment groups

![Chart showing blood urea and BUN levels](chart.png)

Fig 2.7 The increase in blood urea and blood urea nitrogen levels in diabetic group which decreases on 1, 25 dihydroxy vitamin D₃ supplementation.
COMET assay

Fig 2.8 Liver hepatocytes showing DNA damage in diabetic state and recovery after treatment of 1, 25 dihydroxy Vitamin D₃.

Fig 2.9 Pancreatic cells showing DNA damage in diabetic state and recovery after treatment with 1, 25 dihydroxy vitamin D₃.
### Tail lengths

<table>
<thead>
<tr>
<th>Name of the group</th>
<th>Treatment</th>
<th>Average tail length of liver cells (in μm)</th>
<th>Average tail length in pancreatic cells (in μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Saline</td>
<td>7.65 ± 2.20</td>
<td>6.66 ± 1.20</td>
</tr>
<tr>
<td>Diabetic</td>
<td>Alloxan</td>
<td>30.41 ± 2.50</td>
<td>32.45 ± 2.87</td>
</tr>
<tr>
<td><strong>Diabetic + vitamin D</strong></td>
<td><strong>1,25(OH)_2 vitamin D_3</strong></td>
<td><strong>21.80 ± 2.40</strong></td>
<td><strong>19.25 ± 1.90</strong></td>
</tr>
</tbody>
</table>

Table 2.1: DNA tail length of liver and pancreatic tissue observed by alkaline comet assay
Histopathological changes in liver, kidney and pancreas of different treatment groups

<table>
<thead>
<tr>
<th>Tissue Changes</th>
<th>Control Group</th>
<th>Diabetic Group</th>
<th>Diabetic+ vitamin D Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Liver)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Accumulation of lipid droplets</td>
<td>_</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Increased fibrous content</td>
<td>_</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Destruction of bile duct</td>
<td>_</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Hepatocytic degeneration</td>
<td>_</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>a. Centrilobular</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Kidney)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thickening of glomerular basement membrane</td>
<td>_</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Edema in Proximal convoluted tubule</td>
<td>_</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Tubulointerstitial inflammation</td>
<td>_</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>(Pancreas)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Islet cell population</td>
<td>normal</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Insulitis</td>
<td>_</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

Table 2.2: The histopathological changes in the liver, kidney and pancreas of diabetic and 1,25 dihydroxy vitamin D₃ treated mice
Histopathological changes in hepatocytes of different treatment groups

Fig 2.10 Shows the histopathological changes in the hepatocytes of control, diabetic and vitamin D₃ supplemented groups.

Histopathological changes in kidney tissues of different treatment groups

Fig 2.11 Shows the histopathological changes in the kidney tissues of control, diabetic and vitamin D₃ supplemented group.
Histopathological changes in pancreatic tissues of different treatment groups

Fig 2.12 Shows the histopathological changes in the pancreatic tissues of control, diabetic and vitamin D supplemented group.
GLUT-4 expression levels in different treatment groups

Fig 2.13 shows GLUT-4 transporter expression determined by sandwich ELISA in different treatment groups (control, diabetic and vitamin D supplemented ones)