Effect of 1, 25 dihydroxy vitamin D$_3$ in combination with ascorbic acid on type 2 diabetes mellitus.
1 Glucose Homeostasis

1.1 Fasting blood glucose

Fig.1 shows the fasting blood glucose levels and serum calcium levels in all four groups (Control, Diabetic, Diabetes + Vitamin C, Diabetes + Vitamin C+ vitamin D). The FBG is seen to be increased in the group with diabetes 250±0.41 mg/dl as compared to the control group 160.97±0.21 mg/dl, marking the proper establishment of diabetic state. The group with diabetes, when given vitamin C supplementation shows a decrease in the FBG levels 210±0.37 mg/dl, and when only vitamin D was administrated the level reached was 190±0.37 mg/dl however, the group given the combination of vitamin C+D was found to reduce the FBG to levels similar to group given vitamin D alone thus demonstrating that the reduction is majorly due to the presence of vitamin D in the combination 190±0.40 mg/dl and vitamin C seems to have little effect on hyperglycemia.

Fig.3 also depicts the serum calcium level of all four groups. In control group the level was found to be (12.64±0.001 mg/ml). However, a marked decrease is seen in the combination group (vit. C+D) (9.5±0.40 mg/ml) in comparison to the decreased levels in diabetic group (7±0.43 mg/ml) which is symptomatic of function of vitamin D present in the combination (vit. C+D). However, vitamin C supplemented group did not show any change in the serum calcium levels.

1.2 Activities of glucokinase or hexokinase IV, glucose-6-phosphatase and fructose 1,6 bisphosphatase

The hexokinase activity (fig 3.1) was 0.744±0.002 μg/mg/ml and 0.523±0.004 μg/mg/ml in control liver and kidney which was reduced to 0.241±0.014 μg/mg/ml and 0.175±0.032 μg/mg/ml in the diabetes group. Vitamin C supplementation resulted in a very small recovery of 0.2842±0.003 μg/mg/ml and 0.274±0.024 μg/mg/ml while on combination treatment with vitamin C+ vitamin D, the activity recovered significantly 0.701±0.003 μg/mg/ml and 0.465±0.004 μg/mg/ml which was comparable to vitamin D alone 0.717±0.003 μg/mg/ml and 0.47±0.002 μg/mg/ml (Fig.2). Further the activities of G6Pase and FBPase (fig 3.2, 3.3) were analyzed in the liver and kidney of the control group and were found to be 1.153±0.02 μg/mg/ml; 0.69±0.032 μg/mg/ml and 0.1071±0.002 μg/mg/ml; 0.596±0.012 μg/mg/ml
respectively. However, there was a significant increase in their activities in the group with diabetes, the FBPase activity was estimated to be 0.2693±0.004 μg/mg/ml; 0.938±0.023 μg/mg/ml and G6Pase activity was found to be 2.918±0.008 μg/mg/ml; 1.05±0.0035 μg/mg/ml, showing a rapid enhancement of glycogen breakdown, indirectly demonstrating an impairment in the amount of glucose reaching the cell. Their activity levels recovered to 0.13733±0.008 μg/mg/ml; 0.365±0.019 μg/mg/ml in case of FBPase and 1.0546±0.01 μg/mg/ml; 0.77±0.035 μg/mg/ml in G6Pase respectively on vitamin C + vitamin D supplementation. In case of vitamin C supplementation, the recovery of both the enzymes was not significant. However, in the group treated with vitamin D alone there was a significant change with activity levels of FBPase 0.2733±0.008 μg/mg/ml; 0.0126±0.003 μg/mg/ml and G6Pase 0.71±0.01 μg/mg/ml; 0.75±0.04 μg/mg/ml.

2 oxidative stress

2.1 Activities of superoxide dismutase, catalase, glutathione-S-transferase

Fig. 3.4, 3.5, 3.6 represents the antioxidant enzyme parameters with the activities of SOD, catalase (CAT) and GST in liver and kidney. In control group the activities of SOD, catalase and GST were 96.58±0.043 μg/mg/ml; 75.8±0.021 μg/mg/ml, 3.9±0.4 μg/mg/ml in liver and 1.7±0.0019 μg/mg/ml; 80.37±0.46, 37.66±0.001 μg/mg/ml in kidney respectively. However, in case of diabetes all the enzyme activities exhibited a decline. Vitamin C supplementation group exhibited a marked increase SOD 85.65±0.4 μg/mg/ml; 68.5±0.003 μg/mg/ml, CAT 3.8±0.25 μg/mg/ml; 0.88±0.0023 μg/mg/ml, GST 54.53±0.35 μg/mg/ml; 31.29±0.007 μg/mg/ml, in liver and kidney respectively. In case of combination treatment group the increase in enzyme activities was comparable to vitamin C supplemented group SOD 65.54±0.2 μg/mg/ml; 59.37±0.028 μg/mg/ml, CAT 3.059±0.33 μg/mg/ml; 0.81±0.0022 μg/mg/ml and GST 43±0.27 μg/mg/ml; 28.75±0.0019 μg/mg/ml suggesting that vitamin D has almost no role in directly controlling oxidative stress.

2.2 Levels of glutathione, sulphydryl group and malondialdehyde

In case of type 2 diabetic mice the levels of both these cellular reductants were found to be greatly reduced in liver (fig 3.7) GSH 0.1121 ± 0.03 nmol/gm tissue and SH
0.1015±0.015 nmol/gm tissue as compared to normal mice GSH 0.1845 ±0.0105 nmol/gm tissue and SH 0.2451±0.0345 nmol/gm tissue. These levels were though raised with vitamin C supplemented group GSH 0.1645±0.03 nmol/gm tissue; SH 0.2348±0.0144 nmol/gm tissue but the increase was more pronounced with combination of vitamin C and vitamin D suggesting the effectiveness of the treatment. In case of lipid peroxidation, MDA levels were much higher in diabetic group 0.8940±0.0153 nmol/mg protein, as compared to control group 0.3940±0.0180 nmol/mg protein. Vitamin C supplementation helped in decreasing the MDA levels 0.44±0.0189 nmol/mg protein, yet the results were found to be better with combination treatment 0.394±0.0208 nmol/mg protein.

3 Estimation of liver function markers (LFT) in serum

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

The glutamate pyruvate transaminase (GPT) or alanine transaminase (ALT) and glutamate oxaloacetate transaminase (GOT)/ aspartate transaminase (AST) levels were increased in diabetes 171.40± 0.42 U/L and 167.39±0.45U/L while alkaline phosphatase (ALP) was not affected throughout. The recovery of GPT and GOT was better with vitamin C supplementation with 109.5 ± 0.28 U/L, GPT and 120.96±0.48 µg/mg/ml, GOT respectively as compared to vitamin D alone with 80±.254 U/L, GPT and 95.64±0.28 U/L, GOT. However, in case of combination supplemented group with both ascorbic acid and 1, 25 dihydroxyvitamin D₃ the levels of enzymes reached to GPT; 123.25±0.33 U/L and GOT; 154±0.25 U/L.

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± 0.034 mg/100ml) which was increased in case of diabetic group to (0.04317±0.022 mg/100ml), however on vitamin D supplementation the creatinine levels were reduced to (0.038±0.033 mg/100ml) compared to groups given vitamin C (0.3917±0.046 mg/100ml) and Vit.D+Vit.C (0.03916±0.021 mg/100ml). The combination thus is most effective in bringing down serum creatinine levels closer to the control group (fig 3.9).
4.2 Blood urea and BUN (Blood urea nitrogen)

The levels of blood urea and BUN (fig 3.10) in control group were estimated to be 16.71±0.41 mg/dl, 35.759±0.29 mg/dl whereas in case of diabetic group it was 21.8±0.17 mg/dl, 46.652±0.36 mg/dl both these parameters depicted a significant increase, however on supplementation with vitamin D the levels were almost normalised to (21.8±0.32 mg/dl and 46.654±0.44 mg/dl) as compared to group given vitamin C (17.4485±0.38 mg/dl, 37.339±0.25 mg/dl) and Vit.C+Vit.D (16.9755±0.44 mg/dl, 36.82957±0.37 mg/dl) supplementation. Increase in urea is indicative of negative nitrogen balance which is an index of chronic illness and muscle wasting.

5 DNA damage

Diabetes affected the major organs (liver, kidney and pancreas) adversely. The tissue damage was monitored using the COMET assay of the major tissues. In this assay the migration of DNA from nucleus is expressed in terms of tail length or formation of comet which is a direct measure of DNA damage. The DNA tail length of diabetic liver and pancreatic cells was 30.41 ± 2.50 µm and 32.45± 2.87 µm respectively while in control group the tail length was 7.65 ± 2.20 µm and 6.66 ± 1.20 µm. Vitamin C supplemented group showed a partial recovery exhibited by decrease in liver and pancreatic DNA tail length with values of 21.80 ± 2.40 µm and 19.25 ± 1.90 µm respectively while in case of vitamin C+ vitamin D supplemented group the change in the DNA damage was more evident as seen by the shortening of DNA tail length in liver to 18±2.1 µm and pancreas 16±2.34 µm whereas in vitamin D alone treated group, it was 21.80 ± 2.40 µm and 19.25 ± 1.90 µm (Table 3). Therefore, the effect of vitamin C and vitamin D appears synergistic.

6 Histopathological analysis

The histopathological analysis revealed increased accumulation of lipid droplets, characteristic of non-alcoholic fatty liver disease in hepatocytes of diabetic group along with increased destruction of bile duct, a marker of cirrhosis, a significant increase in fibrous content (fibrosis) and centrilobular hepatocytic degeneration (suggestive of parenchymal tissue cell death) 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.

The groups supplemented with vitamin C alone, vitamin D alone and vitamin C+ vitamin D all the markers of NASH, kidney degeneration and pancreatic tissue damage were found to be decreased, showing similarity with the control group (Table 3.1).

7 Spectroscopic study

Since fluorescence technique is considered more sensitive in order to identify inter and intra molecular interaction, we observed the emission spectra of the vitamins alone and their combination. We observed in fig 3.17a, b that the emission peaks of both vitamin C and vitamin D lies in the range of 350-400nm, and the combination shows maximum emission in this range. Thus indicating no strong interaction between vitamin C and vitamin D.

The FT-IR studies of vitamin C and vitamin D were recorded in ATR mode at room temperature at different time intervals in order to understand the interaction, if any. The characteristics IR bands of free vitamin C at at 1158, 1396, 1690 and 1761 cm \(^{-1}\) were assigned to the stretching and bending vibrations of C=O and C–O hydroxyl groups, respectively. The strong band at 1238 cm \(^{-1}\) was originated due to C–O–C stretching. The peaks present in the region 821–1015 cm \(^{-1}\) were linked with the five membered lactone ring deformation. However, various vibrational observed in the range 3000–3380 cm \(^{-1}\) in the IR spectrum was attributed due to stretches of OH groups. On immediate addition of vitamin C with D, there was no major spectral shifting observed for OH band observed at 3352 cm \(^{-1}\). After 1 h, the positions of OH band shifted towards lower frequencies to 3459 cm \(^{-1}\) (K.Nakamoto, 1997). This shift in the spectra indicates that there was some chemical interaction between vitamin C and D.

8 GLUT-4 expression study

The Glut-4 expression levels were assayed by sandwich elisa in adipocytes and skeletal muscles. It was 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 levels were found to be slightly improved in vitamin C supplemented group adipocytes ; 6.5±0.04ng/ml, skeletal muscles ; 5.257±0.28 ng/ml but the change in level was more significant in the group treated with both vitamin C + vitamin D i.e. adipocytes ; 7.07±0.044ng/ml, skeletal muscles ; 8.72±0.036ng/ml as well as with vitamin D alone, thus depicting the efficiency of the combination in normalizing the GLUT4 levels on the cells (fig 3.11).

**Fasting blood glucose level and serum calcium levels in control, diabetic, vitamin C and vitamin C+vitamin D treated mice.**

![Graph showing fasting blood glucose and serum calcium levels](image)

Fig. 3 Shows the changes in fasting blood glucose and calcium levels in different treatment groups. Treatment detail given in methods.
Hexokinase activity levels in control, diabetic, vitamin C and vitamin C+vitamin D treated mice.

Fig 3.1 Shows the activity levels of Hexokinase in different treatment groups.
FBPase activity levels in control, diabetic, vitamin C and vitamin C+vitamin D treated mice.

Fig 3.2 The activity levels of FBPase in different treatment groups.
G6Pase activity levels in control, diabetic, vitamin C and vitamin C+vitamin D treated mice.

Fig 3.3 The activity levels of G6Pase in C in different treatment groups.
Superoxide dismutase activity levels in control, diabetic, vitamin C and vitamin C+vitamin D treated mice.

Fig 3.4 The activity levels of SOD in different treatment groups.
Catalase activity levels in control, diabetic, vitamin C and vitamin C+vitamin D treated mice.

Fig 3.5 The activity levels of catalase in different treatment groups.
Glutathione-s-transferase activity in control, diabetic, vitamin C and vitamin C+vitamin D treated mice.

Fig 3.6 The activity levels of GST in different treatment groups.
GSH, SH and MDA levels in control, diabetic, vitamin C and vitamin C + vitamin D treated mice.

Fig 3.7 The levels of GSH, SH and MDA in different treatment groups.
**SGOT, SGPT and ALP activity levels in control, diabetic, vitamin C and vitamin C+vitamin D treated mice.**

Fig 3.8 The levels of SGOT, SGPT and alkaline phosphatase in different treatment groups.
Serum creatinine levels in control, diabetic, vitamin C and vitamin C+vitamin D treated mice.

Fig 3.9 The levels serum creatinine in different treatment groups.
Blood urea and BUN levels in control, diabetic, vitamin C and vitamin C+vitamin D treated mice.

Fig 3.10 The levels of blood urea and BUN in different treatment groups.
GLUT-4 expression levels in control, diabetic, vitamin C and vitamin C+vitamin D treated mice.

Fig 3.11 The levels GLUT-4 expression levels in different treatment groups.
Fig 3.12 Liver hepatocytes showing DNA damage in diabetic state and recovery after treatment of 1, 25 dihydroxy vitamin D₃, vitamin C alone and vitamin C along with 1, 25 dihydroxy vitamin D₃.
Fig 3.13 Pancreatic DNA damage in diabetic state and recovery after treatment of 1, 25 dihydroxy vitamin D₃, vitamin C alone and vitamin C along with 1, 25 dihydroxy Vitamin D₃.
Tail lengths

Table 3: DNA tail length of liver and pancreatic tissue observed by alkaline comet assay

<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>
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<tr>
<td>Diabetic + vitamin D</td>
<td>1,25(OH)_2 vitamin D3</td>
<td>21.80 ± 2.40</td>
<td>19.25 ± 1.90</td>
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<tr>
<td>Diabetic + vitamin C</td>
<td>Ascorbic acid</td>
<td>21.80 ± 2.40</td>
<td>19.25 ± 1.90</td>
</tr>
<tr>
<td>Diabetic + Vitamin C+</td>
<td>1,25(OH)_2 vitamin D3+ Ascorbic acid</td>
<td>18 ± 2.1</td>
<td>16 ± 2.34</td>
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</tbody>
</table>
Histopathological changes in control, diabetic, vitamin C and vitamin C+vitamin D treated mice.

Table 3.1: The histopathological changes in the liver, kidney pancreas of control, diabetic and treated mice.

<table>
<thead>
<tr>
<th>Tissue Changes (Liver)</th>
<th>Control Group</th>
<th>Diabetic Group</th>
<th>Diabetic+ vitamin C Group</th>
<th>Diabetic+ vitamin C+ vitamin D Group</th>
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</thead>
<tbody>
<tr>
<td>Accumulation of lipid droplets</td>
<td></td>
<td>+++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Increased fibrous content</td>
<td>_</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Destruction of bile duct</td>
<td>_</td>
<td>+++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hepatocytic degeneration a.Centrilobular</td>
<td>_</td>
<td>+++</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Tissue Changes (Kidney)</th>
<th></th>
<th></th>
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<th></th>
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</thead>
<tbody>
<tr>
<td>Thickening of glomerular basement membrane</td>
<td>_</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Edema in proximal convoluted tubule</td>
<td>_</td>
<td>+++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tubulointerstitial inflammation</td>
<td>_</td>
<td>++</td>
<td>+++</td>
<td>+</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Tissue Changes (Pancreas)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Islet cell population</td>
<td>normal</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Insulitis</td>
<td>_</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
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Histopathological changes in hepatocytes of in control, diabetic, vitamin C and vitamin C+vitamin D treated mice.

Fig 3.14 Histopathological changes in the hepatocytes of control, diabetic and vitamins supplemented groups.
Histopathological changes in kidney tissues of in control, diabetic, vitamin C and vitamin C+vitamin D treated mice.

Control

Diabetic

Diabetic+ 1,25 dihydroxy vitamin D₃

Diabetic+ Vit.C

Diabetic+Vit.C+Vit.D

Fig 3.15 The histopathological changes in the kidneys of control, diabetic and vitamins supplemented groups
Histopathological changes in pancreatic cells of control, diabetic, vitamin C and vitamin C+vitamin D treated mice.

Fig 3.16 The histopathological changes in the pancreas of control, diabetic, 1,25 dihydroxy vitamin D₃, vitamin C alone and vitamin C+ vitamin D supplemented groups.
Fig 3.17 Fluorescence spectra of 50μm vitamin D, vitamin C and their combination at 0hr., 1hr. respectively.
FTIR analysis of vitamin C, vitamin D and vitamin C+vitamin D at 0 hours.

Fig 3.18 (a) 1,25 dihydroxyvitamin D₃ alone; (b) vitamin C alone; (c) 1,25 dihydroxyvitamin D₃ and vitamin C combination 50μM each at 0 hr, where X axis denotes wave number (cm⁻¹) and y axis denotes % transmittance.
FTIR analysis of vitamin C, vitamin D and vitamin C+vitamin D after 1 hours.

Fig 3.19 (a) 1,25 dihydroxyvitamin D3 alone; (b) vitamin C alone; (c) 1,25 dihydroxyvitamin D3 and vitamin C combination 50μM each at 1 hr, where X axis denotes wave number (cm$^{-1}$) and y axis denotes % transmittance.