Effect of 1, 25 Dihydroxy Vitamin D$_3$

in Combination with Retinyl Palmitate

on Type 2 Diabetes Mellitus
1 Glucose homeostasis

1.1 Fasting blood glucose

Fig. 4 shows the fasting blood glucose levels in the experimental groups (control, diabetic, diabetes +vitamin A, diabetes +vitamin A + vitamin D). There is a marked increase in the FBG levels of the diabetic group 250.85±0.42 mg/dl as compared to the control 100.65±0.19 mg/dl indicative of the establishment of diabetes. A heightened decrease in the FBG levels can be seen in the diabetic group treated with vitamin A 200.19±0.41 mg/dl; whereas the treatment group provided with an Vit.A+Vit.D combination therapy shows the FBG levels 198.0±0.21 mg/dl close to control however the most significant decrease was observed in group given vitamin D alone 190±0.37 mg/dl thus elucidating the role of 1,25 dihydroxyvitamin D₃ in diabetic treatment.

1.2 Serum calcium estimation

Fig 4.1 depicts the serum calcium level of all four groups. In control group the level was found to be 11.46±0.39 mg/ml. However, the decrease seen in the combination group (Vit.A+Vit.D) 10.01±0.11 mg/ml is comparable to 15.53±0.25 mg/dl in vitamin D treated group 9.5±0.34 mg/ml supporting the role of vitamin D in controlling serum Ca^{2+} levels. However, vitamin A supplemented group did not exhibit any change.

2 Activities of Glucokinase or Hexokinase IV, Glucose-6-phosphatase and Fructose 1,6 bisphosphatase

Fig 4.2, 4.3 and 4.4 shows the changes in the activities of hexokinase, FBPase and G6Pase in the experimental groups. The hexokinase activity was severely affected in the diabetic group in both liver 0.0294±0.023 µg/mg/ml and kidney 0.0149±0.021 µg/mg/ml, with liver showing a greater decrease in the series. Supplementation of Vit.A to the dose tested lead to a recovery L=0.099±0.029 µg/mg/ml, K=0.0131±0.29 µg/mg/ml while supplementation with Vit.A+Vit.D L=0.09±0.046 µg/mg/ml, K=0.01435±0.043 µg/mg/ml resulted in activity levels not as good as vitamin A alone, however, the levels were best recovered with the treatment of vitamin D alone L=0.1033±0.224 µg/mg/ml, K=0.0153±0.003 µg/mg/ml.
FBPase activity follows an inverse pattern. It is elevated in the diabetic group \( L=0.4911\pm0.001 \mu g/mg/ml, K=0.1373\pm0.0647 \mu g/mg/ml \) as compared to the control \( L=0.1032\pm0.008 \mu g/mg/ml, K=0.0893\pm0.00096 \mu g/mg/ml \) but supplementation with both in vitamin A \( L=0.3907\pm0.023 \mu g/mg/ml, K=0.1087\pm0.1044 \mu g/mg/ml \) and Vit.A+Vit.D \( L=0.4279\pm0.329 \mu g/mg/ml, K=0.1151\pm0.0066 \mu g/mg/ml \) resulted in a significant decrease in its activity, with maximum change witnessed in vitamin D treated group \( L=0.2733\pm0.008 \mu g/mg/ml, K=0.1039\pm0.29 \mu g/mg/ml \).

The activity of G6Pase enzyme in control group was found to be; \( L=0.533\pm0.02 \mu g/mg/ml, K=1.156\pm0.032 \mu g/mg/ml \) however, in diabetic group the level was increased to \( 0.918\pm0.008 \mu g/mg/ml, K=0.18\pm0.041 \mu g/mg/ml \) showing a rapid enhancement of gluconeogenesis. On vitamin D supplementation, the activity was found to be significantly decreased; \( L=0.71\pm0.01 \mu g/mg/ml, K=0.129\pm0.033 \mu g/mg/ml \) while in groups given vitamin A supplementation and combination therapy (Vit.A+Vit.D), the enzyme levels were \( L=0.806\pm0.034 \mu g/mg/ml, K=0.15\pm0.29 \mu g/mg/ml \) and \( L=0.8941\pm0.044 \mu g/mg/ml, K=0.17\pm0.43 \mu g/mg/ml \). The results therefore clearly points towards Vit.D as the most effective strategy for controlling the activity of G6Pase.

3 Oxidative stress

3.1 Activities of superoxide dismutase (SOD), catalase, glutathione-s-transferase (GST)

Fig 4.5 and 4.6 represents the changes in the activities of the antioxidant enzymes in control, diabetic, vitamin D, vitamin A and Vit.A+Vit.D supplemented groups (measured in units/mg/protein). There is increase in catalase activity; Liver = \( 59.286 \pm 0.026 \), Kidney = \( 47.93 \pm 0.542 \) in the diabetic group as compared to the control group Liver = \( 30.532 \pm 0.033 \), Kidney = \( 27.7415 \pm 0.053 \), which upon supplementation with vitamin A decreased, catalase ; Liver = \( 44.373 \pm 0.039 \), Kidney = \( 38.049 \pm 0.495 \) however, the decrease was more significant and pronounced in case of group given combination of Vit. A + Vit. D; Liver = \( 1.685 \pm 0.0583 \), Kidney = \( 39.247 \pm 0.495 \). Thereby showing that the combination has a better effect on enzyme activity than individual vitamin.
A similar pattern is followed by SOD and GST in kidney, there is an increase in the activity (SOD; 21.504±0.024 and glutathione-s-transferase; 12.658±0.0557) while in diabetic condition (SOD; kidney=66.057±0.035 GST; kidney=82.203±0.085) which is partially recovered by treatment with vitamin A; (kidney=41.589±0.042, GST; kidney=39.749±0.0668) while in case of Vit.A+Vit.D combination it is (SOD; kidney=20.314±0.0189,GST; kidney=36.427±0.03824), the values obtained were closer to the control in comparison Vit.A alone, suggesting that the combination of Vit.A+Vit.D works more efficiently than Vit.A or Vit.D alone in controlling oxidative stress induced by diabetes.

However, a different pattern was observed in case of liver SOD and GST levels. There was a marked decrease in the enzyme activities of the diabetic group, (SOD; liver=183.272±0.0332 GST; liver=66.144±0.0245) as compared to the control values (SOD; liver=203.426±0.0367, GST; liver=110.009±0.041). Treatment with vitamin A alone and in combination Vit.A+Vit.D resulted in an increase in the SOD values, (vitamin A; liver=190.86±0.0459, vitamin A+D; liver=198.75±0.045). In case of GST however for unknown reason, vitamin A supplementation further reduced the GST levels to 35.53±0.027 while combination of Vit.A+Vit.D elevated the levels closer to the control that is 94.495±0.032. Therefore, all the results of the antioxidant enzymes suggest that supplementation of Vit.A+Vit.D is more effective in controlling oxidative stress as compared to Vit.A or Vit.D alone. (Fig.6,7)

3.2 Activity of glutathione reductase (GR) along with sulphydry (-SH) and glutathione (GSH) level estimation

There was an increase in glutathione reductase activity (fig 4.7) (Liver=0.01362±0.0109, Kidney=0.0108±0.00672) in the diabetic group compared to the control (Liver=0.00506±0.00113,Kidney=0.00723±0.00341) which upon supplementation with Vitamin A; GR was liver=0.00953 and kidney=0.0088, while in the combination treated group GR was (Liver=0.00482±0.00849 kidney=0.00692±0.00116), showing a better recovery of enzyme activity thereby bringing it close to the control group , (GR; liver=0.00506±0.00113, kidney=0.00723±0.00341) as compared to Vit.A or Vit.D alone.
4 Estimation of liver function markers (LFT) in serum

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

The enzyme levels in diabetic and control groups were found to be 171.40± 0.42 U/L and 67± 0.34 U/L respectively and the group given vitamin A supplementation showed the enzyme level of 68.53±0.37 U/L whereas the group with supplementation of both Vit.A+Vit.D was found to have enzyme level 124.38±0.29 U/L while vitamin D supplemented group has (80.3±0.254 U/L),(fig 4.11)

4.2 Glutamate oxaloacetate transaminase (GOT) or aspartate transaminase (AST)

Serum aspartate transaminase is a disease marker enzyme of liver. Its level was found to be increased in case of diabetic group (167.39± 0.45 U/L) as compared to control group (86.521±0.39 U/L) while vitamin A supplemented group was found to have (109.67±0.44 U/L) enzyme level. However, in group given combination therapy Vit.A+ Vit.D the level was estimated to be (110±0.35 U/L) while in case of vitamin D treatment, the level was found to be (95.64±0.28 U/L).

4.3 Alkaline phosphatase (ALP)

In diabetic group the level of ALP was found to be (12.124±0.25 KA units) whereas in control group the enzyme level was (10.694 ±0.37 KA units). In case of vitamin A supplemented group, the level was found to be (11.392±0.47 KA units). The group with Vit.A+Vit.D supplementation was found to have (11.959±0.39 KA units). The most significant recovery was seen with vitamin D with the level of enzyme being (11.12±0.28 KA units). All the results of LFT point towards Vit.D as best supplement for diabetes (fig 4.11)

5 Estimation of renal function markers (RFT) in serum

5.1 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 (0.0411±0.022 mg/100ml), however on vitamin D supplementation the creatinine level was reduced to (0.038±0.033 mg/100ml) compared to groups given vitamin A (0.0391±0.046 mg/100ml) and Vit.A+Vit.D (0.0371±0.021 mg/100ml). The combination thus is most
effect in bringing down serum creatinine levels became closer to the control group (fig 4.12).

5.2 Blood urea and BUN (Blood urea nitrogen)

The levels of blood urea and BUN (fig 4.13) 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 17.54±0.32 mg/dl and 37.53±0.44 mg/dl as compared to group supplemented with vitamin A (18.32±0.38 mg/dl, 39.222±0.25 mg/dl) and Vit.A+Vit.D (19±0.44 mg/dl, 36.38±0.37 mg/dl). Increase in urea is indicative of negative nitrogen balance which is an index of chronic illness and muscle wasting.

6 DNA damage

Diabetes is known to cause major DNA damage in pancreatic cells. The results of comet assay, given in fig 4.14 and 4.15, are indicative of the same. The diabetic group is showing an increase in the DNA tail length; in case of pancreas 32.45±2.87 μm, liver 29.6±1.2 μm as compared to control; pancreas 6.66±1.20 μm, liver 7.1±0.9 μm. A marked decrease in the tail length was observed in the treatment group supplemented with vitamin A alone with pancreas 19.25±1.9μm, liver 25.4±1.33 μm, however, the combination treated group (Vit.A+Vit.D) demonstrated a recovery. It was 26±1.5 μm in pancreas, 22.36±2.8 μm in liver. However, in vitamin D supplemented group the tail lengths were found as; pancreas 21.80±2.40 μm and liver 19.25±1.90 μm and thus the recovery was most significant.

7 Histopathological analysis

Fig 4.16, 4.17 and 4.18 show the histopathological results of control, diabetes and various treatment groups. In diabetic group there was an increased accumulation of lipid droplets, characteristic of non-alcoholic fatty liver disease in hepatocytes together with the 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) whereas in group supplemented with vitamin A all the markers of NASH, cirrhosis and fibrosis were improved, showing similarity with the
control group (Table 4.1). In case of kidney, we observed a significant thickening of glomerular basement membrane in diabetic group along with edema of distal convoluted tubule and tubule-interstitial inflammation, all markers of kidney damage, which were found to be reduced in kidney tissues of group given vitamin A supplementation, however the extent of recovery was more in the combination group supplemented with both Vit.A + Vit.D.

8 GLUT-4 expression study

The GLUT-4 expression levels were assayed by sandwich ELISA in adipocytes and skeletal muscles. It was 5.65±0.02 ng/ml in adipocytes; 5.122±0.033 ng/ml in skeletal muscles in diabetic group as compared to 7.643±0.036 ng/ml in adipocytes; 9.02±0.039 ng/ml in skeletal muscles, in control group. However, the GLUT-4 levels were significantly improved in Vit.D supplemented group; 6.9±0.021 ng/ml in adipocytes; 9.0±0.034 ng/ml in skeletal muscles. This was found to be 5.73±0.035 ng/ml in adipocytes, 7.03±0.024 ng/ml in skeletal muscles in Vit.A supplemented group and in combination group treated with (Vit.A+Vit.D), the levels were; 6.07±0.044 ng/ml in adipocytes, 6.92±0.029 ng/ml in skeletal muscles, thus suggesting the role of vitamin D in normalizing the GLUT-4 expression on the cells (fig 4.22).

6 Spectroscopic study

The absorption spectrum was recorder to show if there was any interaction between vitamin A and vitamin D. Vitamin D shows an absorption maxima at 300 nm which upon immediate addition of vitamin A does not change so as to demonstrate no interaction between the two vitamins i.e. vitamin D and vitamin A.

The FTIR analysis depicted that free vitamin A and D exhibit bands around 1618-1642 cm⁻¹ arising from C-C stretching vibrations (coupled with the neighboring vibrations along the conjugated system). Furthermore, various vibrational bands observed in the region 1200-1500 cm⁻¹ which are connected with the CH₂ scissoring (bending in the plane), twisting or wagging (bending out-of-plan) and the CH deformation modes. Also, the bands in the region 2940-2956 cm⁻¹ corresponded to υ (C-H) stretching vibrations of methyl groups. However, the IR spectra revealed a characteristic broad and strong band in the 3400-3100 cm⁻¹ assigned to the (O-H)
stretching vibration of OH group present in vitamins which is responsible for hydrogen bonding interaction with other molecules. However, the spectral changes are not strong and significant enough to suggest any interaction between vitamin A and vitamin D.

*Fig 4.* shows the fasting blood glucose levels in control, diabetic and treatment groups.
Fig 4.1 shows the serum calcium levels in control, diabetic and treatment groups.
Fig 4.2 shows the hexokinase activity levels in control, diabetic and treatment groups.
Fructose 1,6 bisphosphatase

Fig 4.3 shows the Fructose 1,6 bisphosphatase levels in control, diabetic and treatment groups.
Fig 4.4 shows the Glucose 6 phosphatase levels in control, diabetic and treatment groups.
Fig 4.5 shows the catalase activity levels in control, diabetic and treatment groups.
Superoxide dismutase

Fig 4.6 Depicts the superoxide dismutase activity levels in control, diabetic and treatment groups.
Fig 4.7 Demonstrates the Glutathione-s-transferase activity levels in control, diabetic and treatment groups.
Fig 4.8 shows the Glutathione reductase activity levels in control, diabetic and treatment groups.
Fig 4.9 shows the GSH and –SH levels in control, diabetic and treatment groups.
Fig 4.10 shows the MDA levels in control, diabetic and treatment groups.
Fig 4.11 shows the SGOT, SGPT and alkaline phosphatase activity levels in control, diabetic and treatment groups.
Fig 4.12 shows serum creatinine levels in control, diabetic and treatment groups.
Fig 4.13 shows the blood urea and BUN levels in control, diabetic and treatment groups.
Fig 4.14 Shows the extent of DNA damage and recovery in pancreatic cells of different groups.
Fig 4.15 Shows the extent of DNA damage and recovery in hepatocytes of different groups.
Fig 4.16 Shows the histopathological changes in the hepatocytes of control, diabetic and vitamin A supplemented groups.
Histopathological analysis

Fig 4.17 Shows the histopathological changes in the kidney tissues of control, diabetic and vitamin A supplemented group.
Fig 4.18 Shows the histopathological changes in the pancreas of control, diabetic, vitamin D alone, vitamin A alone and vitamin A+ vitamin D supplemented groups.
DNA tail lengths

<table>
<thead>
<tr>
<th>Name of group</th>
<th>Treatment</th>
<th>Average tail length (µm) (Pancreas)</th>
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<tbody>
<tr>
<td>Control</td>
<td>Saline</td>
<td>6.66±1.20</td>
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<tr>
<td>Diabetic</td>
<td>Alloxan</td>
<td>32.45±2.87</td>
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<td>Diabetic + vitamin D</td>
<td>1,25(OH)2 vitamin D3</td>
<td>14.75±2.1</td>
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<tr>
<td>Diabetic + Vitamin A</td>
<td>Retinyl palmitate</td>
<td>19.25±1.9</td>
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<tr>
<td>Diabetic +Vitamin A+Vitamin D</td>
<td>Retinyl palmitate + 1,25 dihydroxy vitamin D3</td>
<td>16±1.5</td>
</tr>
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Table 4: 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 (Liver)</th>
<th>Control Group</th>
<th>Diabetic Group</th>
<th>Dibetic+ vitamin A Group</th>
<th>Dibetic+ vitamin D Group</th>
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<tbody>
<tr>
<td>Accumulation of lipid droplets</td>
<td>-</td>
<td>+++</td>
<td>++</td>
<td>+</td>
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<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</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>a. Centrilobular</td>
<td></td>
<td>+++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>b. Perportal</td>
<td></td>
<td>+</td>
<td>_</td>
<td>_</td>
</tr>
<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>
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<td>+++</td>
<td>++</td>
<td>+</td>
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<tr>
<td>Tubulointerstitial inflammation</td>
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<td>++</td>
<td>+</td>
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<table>
<thead>
<tr>
<th>Tissue Changes (Kidney)</th>
<th>Control Group</th>
<th>Diabetic Group</th>
<th>Dibetic+ vitamin A Group</th>
<th>Dibetic+ vitamin D Group</th>
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<tbody>
<tr>
<td>Islet cell population</td>
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<td>+++</td>
<td>++</td>
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<tr>
<td>Insulitis</td>
<td>-</td>
<td>++++</td>
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Table 4.1: Shows the changes observed in liver, kidney and pancreas of diabetic and vitamins supplemented mice group with the number of (+) signs linearly indicating the extent of change.
Fig 4.19 represents the UV-absorption spectra of vitamin A and vitamin D alone and in combination at 0 hr. and 1 hr.
FT-IR analysis

Fig 4.20 (a) 1,25 dihydroxyvitamin D₃ alone; (b) vitamin A alone; (c) 1,25 dihydroxyvitamin D₃ and vitamin A combination 50μM each at 0 hr, where X axis denotes wave number (cm⁻¹) and y axis denotes % transmittance.
Fig 4.21 (a) 1,25 dihydroxyvitamin D₃ alone; (b) vitamin C alone; (c) 1,25 dihydroxyvitamin D₃ and vitamin C combination 50μM each at 1 hr and 2 hr respectively, where X axis denotes wave number (cm⁻¹) and y axis denotes % transmittance.
Fig 4.22 Shows GLUT-4 transporter expression determined by sandwich ELISA in different treatment groups including control, diabetic, vitamin A supplemented, vitamin A and vitamin D supplemented ones.