Discussion
Diabetes, a multifaceted disease characterized by high blood glucose level (Antonio Ceriello 2005). It has taken the form of an epidemic in 21st century. (Amos A. McCarty D, Zimmet P 2010) However, in recent decades there have been significant improvements in patient care but substantial numbers of patients are still not achieving the glycemic control levels specified in current guidelines of World Health Organization (1999). Drugs that are presently being used to avert the onset of diabetes are not good enough, on the contrary, providing drugs is pricey and the side effects require cautious monitoring, therefore vitamins offer a promising potential to help in combating diabetes and its complications without showing any side effects.

In type 2 diabetes mellitus (T2DM), insulin is present at normal levels but the tissues do not respond to the hormone. This is caused by decreased sensitivity of target tissues to the metabolic effect of insulin (Brajendra KT, Arvind KS 2006). In type 2 or non-insulin-dependent diabetes mellitus, muscle and fat cells become 'resistant' to the actions of insulin and compensatory mechanisms that are activated in the β-cell to secrete more insulin are not sufficient to maintain blood glucose levels within a normal physiological range and the major players of T2DM are pancreatic β-cell and insulin. (C. B. Wollheim and G.W. Sharp 1981) Since vitamin D plays a role in maintaining calcium homeostasis, it is expected to have a role in T2DM. Therefore, we have tried to see the role of vitamin D in treatment of diabetes.

Vitamin D has been associated with numerous non-skeletal diseases including T2DM for over a decade (Jessica A. Alvarez and Ambika Ashraf 2010). Compared to healthy controls subjects with T2DM have been observed to have significantly lower circulating 25(OH)D concentrations (R. Scragg et al 1995). However, from a pathophysiological standpoint, both vitamin D deficiency and T2DM share the same risk factors including increased adiposity, age and physical inactivity (which may translate to decreased time spent outdoors or reduced sun exposure) (A.W. Norman et al 1980). It has also been found that there occurs a seasonal variation in glucose and insulin concentration (C. J. de Souza and A. H. Meier 1987) which may correlate to the seasonal variation of vitamin D exposure. In spite of all these findings the mechanism which relates vitamin D deficiency and T2DM remains elusive (Chertow B.S. et al 1983). Our present work is an attempt to shed some light on mechanism of action of vitamin D in T2DM systems.
In the present work we demonstrated the effect of 1, 25(OH)2D3 on glucose metabolism, calcium homeostasis and oxidative stress in type 2 diabetic mice in addition to its influence on histopathology and GLUT4 transporter expression. In addition to its involvement in alteration of glucose metabolic pathway, here, we report that the treatment of 1,25(OH)2D produced significant changes in blood glucose and calcium concentration as well as in the activities of enzymes of glucose metabolic pathway as compared to diabetic group without supplementation. These changes are in accordance with the studies showing the lowering of serum glucose and an increase in serum calcium upon vitamin D supplementation (A. G. Pittas et al 2007). Vitamin D deficiency causes an increase in PTH (K. C. R. Baynes et al 1997) leading to an increase in intracellular calcium (De Boland AR, Norman AW 1990) thus lowering its level in plasma whereas vitamin D supplementation re-establishes calcium homeostasis.

The explanation for reduced levels of FBG and increased serum calcium in vitamin D treated diabetic groups as compared to untreated diabetic group can be given on the basis of its molecular action as well as its role in maintaining calcium homeostasis. Since, 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 also the ability of insulin-target cells to sense the brisk intracellular calcium fluxes necessary for insulin action, such as glucose transport (D. S. Worrall and J. M. Olefsky 2002). This results in peripheral insulin resistance and thus increased plasma blood glucose levels. The mode of action of vitamin D is such that, it binds to its receptor (VDR) on the surface of pancreatic β-cells (Vidal M, Ramana CV, Dusso AS 2002). The ligand bound receptor then enters the cell and forms a heterodimer with retinoid X receptor (RXR) (Roberto Lin and John H. White 2003). This complex may then bind to the vitamin D response elements (VDRE) present in insulin gene promoter (Christakos S, Barletta F, Huening M et al 2003, Maestro B, Davila N, Carranza MC, Calle C. 2003) thereby increasing its transcription and finally increasing the levels of insulin in plasma. This increased insulin secretion can further be a cause of decreased FBG levels in vitamin D treated group.
Another mechanism by which 1,25 (OH)2D3 might lower FBG is suggested by its role in maintaining intracellular calcium concentration. Adequate insulin secretion and sensitivity both are influenced by intracellular calcium concentration (Zemel MB 1998). Therefore, the increase in serum calcium levels in vitamin D3 supplemented group is suggestive of the indirect effects of Vitamin D3 on insulin secretion and sensitivity (Bourlon PM, Faure-Dussert A, Billaudel B 1997), which may be mediated via its well-recognized role in regulating extracellular calcium and calcium flux through the β-cell or by its genomic actions.

Our estimations of the enzyme activities of hexokinase, G6Pase and FBPase showed a distinct pattern. The decreased hexokinase activity in the group with diabetes, is an evidence of the fact that there is insufficient amount of glucose reaching the liver even in the state of hyperglycemia (Glinsman, W. H., E. P. Hem, and A. Lynch 1969), whereas increased activities of G6Pase and FBPase relates to almost twofold increase in gluconeogenic pathway which contribute to increased hepatic glucose production and thus hyperglycemia (Magnusson, I. et al 1992). However, the activities of these enzymes were found to have a reverse pattern in vitamin D3 treated group showing an increase in hexokinase and a decrease in G6Pase and FBPase activities, thereby providing an insight on the effect of vitamin D3 on glucose metabolism which eventually leads to maintenance of glucose homeostasis (Consoli, A. et al 1989). These results confirm the anti-hyperglycemic properties of 1,25(OH)2D3. However it showed no direct effect on the activity of enzymes related to oxidative stress like SOD, catalase, GST.

In the present study we report that the supplementation of vitamin D3 caused significant recovery from the diabetes induced tissue damage (liver and pancreas) when compared to group with no supplementation. We demonstrated it by comet assay and histopathological analysis of the tissues (pancreas, liver and kidney). We observed the shortening of nuclear DNA tail length in the treated group. The most valued explanation of vitamin D3 induced recovery shown by the supplemented group can be on the basis of immune-modulatory and anti-apoptotic actions of vitamin D3 (Rita Riachy et al 2002, Rita Riachy, D'Herbomez M, Lefebvre J, Pattou F 2001). Recent studies have provided conclusive evidence that pancreatic β cell mass is reduced in T2DM and a probable reason for this reduction can be increased β cell
apoptosis, (Sempoux C, Guiot Y, Dubois D, Moulin P, Rahier 2001), the mechanism of this β-cell death being mediated by hyperglycemia.

Glucose, the key physiological regulator of insulin, induces β-cell proliferation in a concentration dependent manner, (Maedler K, Spinas GA, 2001) however the proliferative capacity is suppressed after prolonged exposure to high glucose concentration. This complication is generally initiated by hyperglycaemic excursions which cause β-cell to produce IL-1β (Maedler K, Sergeev P, 2002) followed by FAS upregulation. Under normal conditions, FAS upregulation leads to cell proliferation in the presence of FLIP but in presence of hyperglycaemic excursions, the level of FLIP decreases finally switching the pathway to deleterious signals, causing cell death and eventually diabetes.

This is the site where vitamin D3 might exert its effect and thereby stop apoptosis. Previous studies have shown that the inhibition of β cell function (insulin synthesis and insulin secretion) induced by IL-1β or IFN-γ in vitro is prevented by 1,25(OH)2D3 (Sandler S, Buschard K, Bendtzen K, 1994). Therefore, our results of comet assay are in accordance with the studies showing anti-apoptotic effect of vitamin D3 on pancreatic β cells.

![Diagram](image)

*Fig.5 Hypothetical model illustrating the consequence of hyperglycemia on β-cell production of IL-1β in parallel with insulin secretion. The paracrine effect of IL-1β induces Fas engagement, which in the presence of FLIP leads to β-cell proliferation, differentiation, and increased function.*
The explanation for shortening of nuclear DNA tail length in hepatocytes of the vitamin D₃ treated group, is based on the fact that patients with type 2 diabetes have increased circulating levels of free fatty acids, leptin and TNF-α, (Van Steenbergen W & Lanckmans S 1995) which are also found to be involved in the pathogenesis of non-alcoholic steatohepatitis (NASH) (Shimabukuro M, Zhou YT, Levi M, Unger RH 1998, Hotamisligil GH & Spiegelman BM 1994), further leading to cirrhosis (liver damage) via fibrosis. However, vitamin D supplementation decreases the cytokine levels through decrease in expression of TNF-α, involved in the development of NASH (Cantorna, M. T. 1998). This may also help in reducing FFA and leptin by improving glucose homeostasis thereby reducing liver cirrhosis as shown by the shortening of nuclear DNA tail length in liver cells in vitamin D supplemented group as compared to diabetic group receiving no vitamin D.

In case of histopathology, the tissues showed significant recovery from damage in vitamin D supplemented group. In diabetic group, the liver showed accumulation of lipid droplets, increased fibrous content, destruction of bile duct and hepatocytic degeneration together with significant thickening of the glomerular basement membrane, oedema of proximal convulated tubule, hyaline deposition, degeneration of distal convulated tubule and tubule-interstitial inflammation. However, the pancreatic tissues demonstrated a decrease in islet cell population and presence of insulitis.

To sum up the effect of 1,25 dihydroxyvitamin D₃ on type 2 diabetic mice, it can be said that 1,25 dihydroxyvitamin D₃ maintains intercellular calcium concentration both in pancreas and peripheral insulin sensitive tissues, thus increasing the calcium mediated GLUT4 transporter expression on the surface of skeletal muscles and adipocytes. This helps in increasing the glucose uptake by the cell and simultaneously removing it from circulation, thus bringing down hyperglycemia. 25 dihydroxyvitamin D₃ also enhances the calcium mediated release of insulin from insulin granules in pancreas thereby increasing the concentration of insulin in blood, and making it available to control the increased blood glucose.

Since hyperglycemia is the main player in the development of diabetic complications and also causes oxidative stress and related problems. By bringing down hyperglycemia and establishing normoglycemia, 1,25 dihydroxyvitamin D₃ plays a
major role in controlling type 2 diabetes and related complications. Therefore, we suggest with caution that 1,25 dihydroxyvitamin D₃ is could be used as an add-on therapy for diabetic treatment, this will help in delaying the diabetes related complications.

In addition to the effect of 1,25 dihydroxyvitamin D₃ in the treatment of diabetes, we also analysed the role of this vitamin (1,25 dihydroxyvitamin D₃) in combination with other vitamins i.e. vitamin C (ascorbic acid) and vitamin A (retinyl palmitate). Inability of 1,25 dihydroxyvitamin D₃ to directly improve the anti-oxidant enzyme parameters led us to study the combinations, as it is not only hyperglycemia alone that needs to be normalised but other contributing factors like oxidative stress, that should also be treated and kept under surveillance for effective management of type 2 diabetes mellitus.

Although the mode of action, the targets acted upon and the mechanism of action of both these vitamins (1,25 dihydroxyvitamin D₃ and vitamin C) is totally different from each other but given together, they have the potential to act upon the two biggest most basic abnormalities of type 2 diabetes mellitus i.e. hyperglycemia and oxidative stress (Anabela Rolo P, Carlos Palmeira M. 2006). In addition to their dual action, they are less costly, easily available, and free from side effects and pose no threat of further complications. Our work compares the effect of vitamin C alone and in combination with vitamin D on glucose metabolic enzymes, anti-oxidant enzymes, DNA damage, histopathological changes and levels of GLUT4 expression in type 2 diabetic mice.

It was found that the group given combination therapy (Vit.C+Vit.D) showed a significant decrease in FBG levels as compared to the group given vitamin C alone. A probable explanation for this finding can be given by looking at the individual actions of these vitamins. Vitamin C, a known anti-oxidant, plays its anti-diabetic role by scavenging the reactive oxygen species (ROS) generated due to hyperglycemia (Misato Kashiba 2002), thus protecting β-cells from free radical mediated damage thereby helping in increased cell survival and near normal insulin secretion eventually bringing down fasting blood glucose levels (Mohammad Afkhami-Ardekani & Ahmad Shojaoddiny-Ardekani 2007). Vitamin D, however adopts a different mode of action,
mediated via calcium homeostasis. Intracellular calcium concentration is very important for insulin secretion and sensitivity as 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 (Worrail DS and Olefsky JM. 2002). Thus, resulting in peripheral insulin resistance and increased plasma blood glucose levels. Vitamin D supplementation therefore maintains the required calcium concentration and help in increased insulin secretion and sensitivity (Bjorklund, Lansner A and Grill VE. 2000) thereby removing excess glucose from circulation. When both these vitamins are given together as (Vit.C+Vit.D) combination they are expected to give a near two-fold effect as evident by results of combination group when compared to vitamin C alone group. This effect is also supported by the observation that serum calcium level is also increased in (Vit.C+Vit.D) combination group, suggesting that calcium mediated anti-hyperglycaemic effect is because of vitamin D alone as in vitamin C treated group, no changes were observed in serum calcium levels.

The enzyme activities of hexokinase, G6Pase and FBPase also showed a distinct pattern. The decreased hexokinase activity in the group with diabetes, depicts insufficient supply of glucose to the liver (Glinsman WH, Hem EP and Lynch A. 1959) however, the increased activities of G6Pase and FBPase are indicative of enhanced hepatic glucose production and finally hyperglycemia (Magnusson I. 1992). However, in the group given combination therapy, the enzymes display improvement in activities compared to the group given vitamin C alone. The combined action of vitamin C+ vitamin D, helps in decreasing β-cell death via scavenging ROS by vitamin C and increase in insulin secretion and sensitivity mediated by vitamin D. Once the required amount of insulin enters circulation, it facilitates glucose to enter into the peripheral tissues, leading to normalization by upregulating hexokinase $\uparrow$ and downregulating G6Pase, FBPase $\downarrow$. The probable reason for a marked changed in the activities in the combination therapy group is that vitamin D not only works on insulin secretion but also on insulin sensitivity (Bourlon PM et al. 1997). This increase in insulin sensitivity may be due to the increased expression of glucose transporter (as expression on the cell surface also depends on intracellular calcium concentration) mainly GLUT-4 in peripheral tissues namely liver and adipocytes. This led to increase
in extracellular glucose uptake that accounts for increase in hexokinase activity with a simultaneous decrease in G6Pase and FBPase activities. The dual action of vitamin C+ vitamin D in (D + Vit.C + Vit. D) group, is therefore, responsible for the improvement in the activities of glucose metabolic enzymes as compared to vitamin C alone group.

We also analysed the antioxidant enzyme activities in both the treatment groups and found that the presence of vitamin C alone as well as with vitamin D, imparts antioxidant effect to the therapy (Reusch JEB et al. 1991). Since it is already known that vitamin D does not possess any antioxidant properties, the enzyme activities of SOD, catalase and GST show a better result in group given vitamin C alone as compared to vitamin C+ vitamin D group. These results can be further understood by observing the levels of cellular reductants (GSH and SH) in groups given vitamin C alone (D+Vit.C) and along with vitamin D (D+Vit.C+Vit.D) (Daniel Tessier M. 2009).

In order to further clarify the direct effect of the vitamin C supplementation alone and in combination with vitamin D we analysed MDA levels to assess the extent of lipid peroxidation. It was observed that though lipid peroxidation was decreased in vitamin C supplemented group (D+Vit.C) (Mehmet Çay et al. 2001, Laranjinha J et al. 1999) but in the combination treated group (D+Vit.C+Vit.D), the reduction in MDA levels was more pronounced, supporting the beneficial effect of the combination in targeting two major players of type 2 diabetes that is hyperglycemia and oxidative stress.

Similar results were obtained in case of DNA damage. It was found that the combination therapy (D+Vit. C+Vit.D) lead to a greater decrease in DNA tail length as compared to vitamin C alone group (D+Vit.C). These results also support the very idea of using both these vitamins (Vit.C+ Vit.D) to target hyperglycemia (Paolisso G et al. 1995) and oxidative stress (Meerza D et al. 2012, Sebastian Padayatty et al. 2003). The histopathological analysis of the liver demonstrated an accumulation of lipid droplets, increased fibrous content, destruction of bile ducts and hepatocytic degeneration in diabetic group while groups treated with vitamin C alone (D+Vit.C) and combination (D+Vit.C+Vit.D) depicted a decrease in these pathological conditions with the extent being same in both the groups. 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 circulations these NEFA’s compete with glucose for oxidation in liver, causing a sequential inhibition of pyruvate dehydrogenase, phosphofructokinase and hexokinase II activity and causing accumulation of diacylglycerol, ceramides and lipids in the liver. These metabolites after accumulation 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 to further insulin resistance (Steven Kahn E. 2006). 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 leading to increased free radical production which ultimately induces inflammation; cellular necrosis and also fibrosis, all contributing to liver damage (Diego Garcia-Compean 2009).

Interestingly, vitamin C supplementation alone and along with vitamin D acts by normalising these complications via two probably distinct pathways. Vitamin C, a potent antioxidant, scavenges the free radicals generated by fatty acids and its metabolites and thus stops the vicious circle of inflammation, cirrhosis and fibrosis, protecting the liver from free radical induced damage. However, in case of vitamin D the site of actions seems to be slightly different. Vitamin D has been found to maintain the calcium homeostasis within the cells which is very important for insulin action, therefore, by maintaining required calcium concentration in adipocytes, vitamin D can increase the insulin sensitivity via increasing GLUT-4 expression and GLUT-4 mediated glucose uptake thereby decreasing lipolysis and NEFA’s levels in circulation, finally stopping NEFA’s induced liver damage.

Therefore, from the analysis of the results, we can conclude that the combination therapy consisting of both vitamin C and vitamin D is likely be more beneficial in correcting the underlying abnormalities compared to any one of these vitamins alone. This combination approach brings together the anti-hyperglycemic effect of vitamin D and the anti-oxidant effect of vitamin C, thus targeting both, the cause and the effect leading to type 2 diabetes and related complications. This, combination therefore, in
all probability will be very effective if given as supplementation to people suffering from type 2 diabetes mellitus.

The next combination of vitamins that we used in this study was vitamin A+ vitamin D.1,25 dihydroxy vitamin D₃ and vitamin A both belong to the class of steroids, mediate their actions both at the cellular as well as at genetic level. The molecular signalling pathway of both of these hormones is however rather complex and discrepancies have been found in the results observed. According to the common notion, the reason for this could be because both of these vitamins mediate their molecular actions via interacting with retinoid x receptor (RXR) present inside the cell. Studies have suggested the antagonistic relationship of vitamin A with vitamin D. However, it is of due importance to note that vitamin D can mediate its actions not only by making vitamin D receptor VDR: RXR heterodimers but also as homodimers (VDR:VDR) (Cheskis B, Freedman LP) or heterodimers VDR:RAR (retinoic acid receptor) and VDR:TR (thyroid receptor), depending upon the type of vitamin D response element (VDRE’S) present in the gene to be upregulated or downregulated (Thompson PD, Jurutka PW). The preferred heterodimers for VDRE’s is: RXR: RXR for (D receptor) DR1 type, VDR: RXR for DR3 type, VDR: TR for DR4 type, VDR: RAR for DR5 type (Cheskis B, Freedman LP). DR1-DR6, are the vitamin D response elements, having direct repeats with 1, 2, 3, 4, 5 and 6 flanking nucleotides.

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\text{e.g. DR5} \quad \text{GGGTCAACGAAAGGTCACT} \\
\text{DR6} \quad \text{GGGTGACCGCAAAGGTGACT}
\]

Therefore, at the molecular level, no specific rule can define the relationship between vitamin A and vitamin D as antagonistic or synergistic as it varies from gene to gene. Also, the type of VDRE’S (DR1, DR3, DR4, DR5 or DR6) will determine whether a highly specific or rather flexible binding of VD in combination with other ligands like 9 cis retinoic acid and all trans retinoic acid can be expected. However, many in vitro studies suggest that 9 cis retinoic acid positively co-ordinates the action of nuclear signalling of vitamin D and both 9 cis retinoic acid and 1,25 dihydroxyvitamin D₃ enhanced reporter gene expression from a vitamin D responsive promoter. (Cheskis B, Freedman LP)
At the cellular level, the mode of action of these vitamins is rather simple to understand. Retinaldehyde, the first intermediate formed from vitamin A, and \textit{all trans} retinoic acid, mediates most of the actions, whereas 1, 25 dihydroxy vitamin D\textsubscript{3}, the final metabolite of vitamin D performs all the functions. Retinaldehyde inhibits PPAR\textgamma activation thus (a) decreasing the triglyceride content in white adipose tissues, skeletal muscles and liver, (b) increasing the effect of leptin and (c) enhancing fatty acid combustion and energy dissipation. The other metabolite, \textit{all trans} retinoic acid acts as a specific ligand for PPAR\beta/\delta, a key regulator of fatty acid oxidation in muscle and fat. Therefore, both these metabolites act together, ameliorating induced obesity and insulin resistance. 1, 25 dihydroxy vitamin D\textsubscript{3}, the functional form of vitamin D, maintains the required calcium levels inside the cells possessing vitamin D receptors (VDR’s) (like pancreas, skeletal muscles and adipocytes), finally increasing the insulin secretion by pancreas and insulin sensitivity in target tissues, as both these processes are calcium dependent.

In the present study the vitamin A supplemented group demonstrated a decrease in FBG levels and FBPlase activity along with an increase in the activity of hexokinase as compared to the diabetic group, suggesting towards the increased amount of glucose reaching liver which may be due to increased expression of GLUT-2 transporter on the surface of the liver cells. Vitamin A supplementation has already been found to induce the expression of GLUT-2 in insulinoma cell line INS-1. All trans-retinoic acid induced GLUT-2 m-RNA expression was observed after 48 hrs of incubation. Another mechanism by which vitamin A leads to increased glucose uptake may involve PKC and thus effecting insulin sensitivity positively. It has been found that all trans retinoic acid modulates the activity of PKC by directly binding to its isozymes (Fernandez-Mejia C, Davison MB). These PKC isoforms might play a role in ser/thr phosphorylation of insulin receptor substrate (IRS-1, IRS-2) and also inactivate NADH oxidase. This may inactivate and block the downstream signalling pathway of insulin action and free radical production. This effect of all trans-retinoic acid may prove helpful in insulin signalling pathway ultimately improving insulin sensitivity and reducing oxidative stress. However, the changes were not that significant in the group given the combination treatment i.e. vitamin A+ vitamin D, this could be due to the presence of vitamin D and since both vitamin D and vitamin
A involve RAR for their cellular action, the presence of both these vitamins together can lead to the decrease in the available RAR in the cell. Once the amount of RAR decreases, neither of the vitamins (vitamin D or vitamin A) are able to carry out their molecular functions, thereby leading to poor results.

Fig 5.1 Suggestive mechanism of action vitamin A involved in bringing down oxidative stress parameters and affecting expression of enzymes.

This is the reason that combination of vitamin D and vitamin A showed mixed results depending upon the parameter analysed and the pathway used by the vitamins. The oxidative stress parameters showed better results in case of combination group when compared to vitamin A alone because to lower down the free radical production vitamin A probably uses the PKC pathway, inhibiting NADH oxidase and thus lowering oxidative stress parameters however in case of glucose metabolic enzymes both vitamin A and vitamin D probably mediate their effects via molecular mechanism causing RAR insufficiency in the cell and eventually effecting the results negatively than in case of either of the vitamins alone.

The oxidative stress parameters observed in our study, with the only exception of SOD showed a marked decrease (catalase, GST and GR) in vitamin A supplemented group whereas there was no significant difference between vitamin A treatment group and the group given vitamin A+ vitamin D. This could be due to the fact that vitamin
D alone did not directly influence oxidative stress parameters while vitamin A has demonstrated anti-oxidant action in our treated groups. The decrease in the activity of SOD in the diabetic condition can be explained on the basis of excessive production of superoxide and hydroxyl radicals, which might have utilized all cellular SOD as the first line of defence against the free radicals. This result could also be attributed to the increase in glycation in case of diabetic group, which inactivate the enzyme. However, vitamin A or retinoic acid causes an increase in the SOD activity by acting as a lipoperoxy scavenger thereby controlling the free radical mediated glycation of SOD, resulting in an increase in SOD activity.

The increase in the MDA levels in vitamin A supplemented group can be explained considering its role in adipocyte metabolism. Vitamin A has an inhibitory effect on adipogenesis together with stimulating fat thermogenesis. The regulatory cascade controlling adipogenesis involves C/EBP followed by increased expression of PPARγ, once induced PPARγ activates the transcription of adipocyte marker genes through interaction of PPARγ: RXR heterodimer (Aria K, Lizuka S, Tada Y, Oikawa K). Vitamin A or retinoic acid inhibits adipogenesis by interfering with the transcriptional activity of C/EBP proteins, blocking the C/EBP mediated induction of downstream genes especially PPARγ. (Chawla A. and Lazar MA) In addition to this, retinoic acid (RA) upregulates RARγ expression and decrease the expression level of RARα thus favouring RAR: RXR heterodimer formation, over PPARγ: RXR heterodimer formation eventually bringing adipogenesis to a halt. (Schwarz EJ, Reginato MJ, Shao D, Krakow SL). However, the pro-thermogenic effect of RA is mediated through increase in the activity of uncoupling protein-1 (UCP-1), which is an inner mitochondrial membrane protein, capable of short circuiting the proton gradient generated by respiratory chain. This uncouples fuel oxidation from ATP synthesis and generates heat (Kawada T, Kamei Y. and Sugimoto E). Therefore, both anti-adipogenic and pro-thermogenic effects together reduce adipogenesis/lipogenesis and enhance lipolysis leading to an increase in circulating levels of lipids and free fatty acids (FFA’s). These metabolites then reach liver and are subjected to mitochondrial oxidation, producing high levels of oxidation products including MDA thus accounting for an increase in MDA levels in vitamin A supplemented group.
On the other hand, the group given combination treatment with vitamin A and vitamin D exhibited lower level. This may be due to the effect of vitamin D in bringing down ROS through controlling hyperglycemia, which is partly responsible for high MDA levels.

It is also known that beta cells contain low level of antioxidant enzymes when compared to other cells and therefore succumb more easily to the oxidative damage thus adversely effecting pancreatic DNA (Nedergaard J, Golozoubova V). In the current study we report that the supplementation of vitamin A has a protective role on the pancreatic tissue damage inflicted by type 2 diabetes. The explanations for this effect is based on the antioxidant activity of retinol on beta cell function and integrity thus leading to a reduction in pancreatic DNA tail length, in vitamin A supplemented group as compared to the diabetic group. Our data suggests cytoprotective effect of vitamin A supplementation and in the treatment of diabetes but further experiments are required to affirm the same and pave the way for a new and effective treatment schedule. The histopathological changes in the liver of diabetic group while a decrease in these pathological conditions was observed when supplemented with Vit.A. The defect in insulin sensitivity leads to increases lipolysis and thus higher levels of circulating non-esterified free fatty acids (NEFA’s). Once in circulation these NEFA’s compete with glucose for oxidation in liver, causing a sequential inhibition of glycolytic enzymes downstream and accumulation of diacylglycerol, ceramides and lipids. These metabolites then activate phosphorylation of insulin receptor substrate 1 and 2 (IRS-1 and IRS-2) finally bringing down the downstream events of insulin signalling pathway and contributing to further insulin resistance (Lenzen S, Drinkgern J, 1996). Another mechanism, by which type 2 diabetes leads to liver damage, is mediated by increased oxidative stress due to mitochondrial dysfunction which ultimately induces inflammation, cellular necrosis and also fibrosis, all contributing to liver damage (Steven Kahn E). In case of kidney, a marked thickening of glomerular basement membrane was observed which is a characteristic feature of glomerulosclerosis. This is formed due to the deposition of glycated proteins along with the presence of edema in distal convoluted tubule and interstitial inflammation, all markers of glomerular-nephritis. However, in vitamin A supplemented group, all these pathological changes were found to be reduced, most probably due to the free
radical scavenging action of vitamin A. Locally generated ROS increase tissue
damage by causing lipid peroxidation, thus destroying the membrane integrity and
causinng tissue damage. However, the dual action of the two vitamins i.e. vitamin
A+vitamin D in the group given combination treatment resulted in better and more
significant changes in the tissues hinting towards the beneficial effect of these two
vitamins. In order to understand the nature of relationship shared by these vitamins
i.e. vitamin A and vitamin D, we carried out FTIR analyses which depicted no
interaction between two vitamins at molecular level thus, there is no clear evidence
that the presence of one vitamin will either be inhibitory to other vitamin or activating
the action of another. What we suspect is that vitamin A and vitamin D share the
receptor VDR-RXR/VDR-RAR, the combination in all probability effect the
downstream signalling pathway. As the distributions of these receptors vary in
different tissues, the effect may be synergistic or antagonistic. Further studies into
gene expression and signalling pathways are however needed to fully elucidate this
effect.

In the end, the study shows that if given in combination, vitamin A and vitamin D can
act together to normalise and bring down type 2 diabetes related oxidative stress and
related complications.