VITAMIN D DEFICIENCY MAY AFFECT THE GLUCOSE UPTAKE IN L6 CELLS BY AFFECTING THE MITOCHONDRIAL METABOLISM

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ABSTRACT

The present study is an attempt to understand the role of vitamin D deficiency in pathogenesis of diabetes and put forth a possible explanation for its mechanism of action. Skeletal muscle cells (L6) were separately grown in media with very low vitamin D as well as optimized dose (10^{-9}M) of vitamin D supplemented media. It was observed that L6 cells grown in presence of vitamin D (10^{-9}M) exhibited high glucose and high calcium uptake as compared to the cells grown in low concentration of vitamin D. As mitochondrial defects are another crucial factor to cause the diabetes and vitamin D is known to improve the mitochondrial metabolism. Hence, in order to study the same, we also determined the activity of NADH oxidase and catalase in all set of experiments. In correlation with calcium and glucose uptake assays, the activity of these enzymes was also found to be improved in presence of vitamin D supplementation. Hence putting all the observations together, the anti-diabetic function of vitamin D can be possibly due to improvement in mitochondrial metabolism.

KEY WORDS: Vitamin D, Diabetes, mitochondria, L6, glucose

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INTRODUCTION

Diabetes mellitus (DM) is one of the major metabolic disorders which manifests clinically as chronic hyperglycemia. The cause of the disease is either insufficient insulin secretion or resistance to insulin in the target tissues. Apart from its well-known “classic” effect in calcium homeostasis and bone metabolism, vitamin D (vitamin D₃, calcitriol, C₂十七H₄₈O₃) has also shown to exert its non-skeletal roles including increased insulin response and expression of glucose transporters, the alteration in any of these factors may lead to diabetes. Vitamin D enhances the insulin secretion from β-cell via regulating the intracellular calcium pool. In peripheral insulin target tissues also, vitamin D enhances insulin sensitivity, possible by transcriptional activation of insulin receptor gene. Another study (2013), correlated increase in GLUT-1 and GLUT-4 glucose transporters gene expression with increased vitamin D concentration in L6 myotubes. Along with these studies, a large number of reports found a reverse relation between vitamin D levels and the risk of DM. Thus, vitamin D can be proposed to be one of the key regulators of glucose metabolism and this is the reason, there is a widespread interest in studying the role of vitamin D in the pathogenesis and prevention of DM. However, the exact mechanism of this link vitamin D and insulin response is incompletely understood. T2DM is characterized by disturbed balance of insulin release and insulin response, and mitochondria is involved in both of these processes. Moreover, as per different studies, mitochondrial dysfunction is also recognized to be an important key factor for the pathophysiology of diabetes. The involvement of mitochondria in causing the diabetes can be explained by claiming that this tiny organelle, being the “powerhouse” of the cell, are devoted to energy production in the form of ATP. Increased ATP/ADP ratio in the cytosol leads to closure of ATP potassium channels, as a result the plasma membrane of pancreatic β cells get depolarized. This change in potential is sensed by calcium channels which then open resulting in influx of calcium. Influx of calcium ions causes secretion of insulin from the insulin-containing vesicles in the β-cell. Hence, ATP; ADP ratio is important in controlling insulin secretion. Similar to vitamin D, mitochondrial defects not only show their effect on insulin secretion and its action on target cells but, it also effects the expression of glucose transporters and glucose uptake as well. This was demonstrated in a study, where the investigators aimed to explore the effect of mitochondrial dysfunction on disturbed glucose homeostasis in adipocytes and they observed a direct correlation between increased ROS production and decreased expression of GLUT4 transporter gene. Impaired activity of two mitochondrial enzymes such as NADH oxidoreductase and citrate synthase has also been detected in muscle biopsy specimens of diabetic patients. Consistent with this, in a microarray study, the master regulator of mitochondrial metabolism i.e. peroxisome proliferator coactivator 1 (PGC1α) was found to be downregulated in skeletal muscles of T2DM patients. Thus, vitamin D deficiency and mitochondrial defects both seem to independently affect the glucose uptake, insulin expression and insulin action. It was not clear until in year 2013, if these two factors are also connected with each other or not, Sinha et al (2013) using human skeletal muscle samples, proposed that vitamin D supplementation may improve the oxidative phosphorylation process. His observations were in consonance with the studies of 1960s and 1970s that led to the recognition that mitochondrial dehydrogenases are activated by calcium ions. So, after considering all the observations from all three different fields of researches, it may be proposed that vitamin D deficiency may possibly cause diabetes by affecting the mitochondrial health. And since, the end result of diabetes is raised levels of plasma glucose; our current study aims to understand the proposed hypothesis by aiming the study of mitochondrial metabolism and glucose uptake in presence and absence of optimum vitamin D concentrations and put forward an explanation for integrated effect of vitamin D deficiency and mitochondrial defect in causing diabetes.

MATERIALS AND METHODS

Cell Line

Rat skeletal muscle cell line (L6) was procured from NCCS (National Centre for Cell Science) Pune, India. Cells were grown and maintained in monolayer culture in Dulbecco’s modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 10mg/ml penicillin, and 100mg/ml streptomycin in an atmosphere of 5% CO₂ at 37°C, until they reached 80-90% confluence.

MTT assay

Cells were seeded at a density of 3x10⁴ cells in 200μl media (DMEM media with 10% FBS) in each well of 96-well plate and incubated for 24 hours. The media with 10% serum was then replaced with 100μl of media with 2% serum and 50μl of 10⁻¹⁰ M-10⁻⁷ M four different concentrations of vitamin D (calcitriol, Windlas Biotech Limited) was supplemented and incubated for next 24 hours. This was followed by addition of 20μl MTT (5mg/ml) reagent to each well. After incubation for 4 hours, reaction was terminated by 100μl of DMSO. The optical density was measured at 570 nm in 96-well plate and percentage cell viability was estimated.

Experimental Design

In order to study the effect of vitamin D on glucose uptake and possible involvement of mitochondria in this process, the different sets of experiments were designed. The first set (Set1) contained cells grown in media with 10% serum, serving as positive control. In the second set (Set2) cells were grown for 24 hrs in media with only 2% serum serving as negative control because serum is the source of vitamin D along with other growth factors. By serum deprivation, the L6 myoblasts also got differentiated in to myotubes which exhibited the highest expression of GLUT4. The third set (Set3) had cells grown in media with 2% serum but supplemented with optimized concentration of vitamin D.
Estimation of Calcium levels
Calcium level detection was done to determine the effect of vitamin D on calcium uptake by L6 cells using LiquiMAX Calcium detection kit in the culture media. Calcium levels for all three sets were determined as per manufacturer’s protocol. Each condition was assayed in three independent experiments in triplicate.

Estimation of Glucose level
Dinitrosalicylic (DNS) colorimetric assay was performed to estimate the glucose levels in the media of different sets of experiments at different time points. For this experimental setup, the 5×10^5 cells per 5 ml of media were seeded in T25 flask for 24 hours in triplicates. Further, 200 µL of the sample was collected at 0 hr, 24 hrs, 48 hrs and 72 hrs and mixed with 200 µL DNS reagent. The solution was heated at 95°C for 30 min and then allowed to cool to stop the reaction. Absorbance was measured at 570 nm in microtitre plate. To study the effect of vitamin D on glucose uptake, the glucose levels were compared amongst all three sets at different time intervals.

NADH oxidase assay
This assay was done in mitochondrial-rich fraction, which was obtained as per standard protocol. NADH oxidase activity was determined as the disappearance of NADH measured at 340 nm in reaction buffer contained NADH (0.2 mM) in Tris-cl buffer (50 mM, pH 7.0). A 10mM NADH stock solution was prepared in 0.1 M HEPES buffer (pH 8.5). The amount of NADH oxidized was estimated by using a molar extinction coefficient of 6.22 x 10^3 M^-1 cm^-1 at 340 nm. Assay was initiated by adding test 45µg of protein sample to 3 ml of assay buffer. Decrease in absorbance was measured for 0 to 8 min and NADH oxidase activity was estimated from the mentioned-below equation. Assuming the NADH oxidase activity of set 1 as 100%, it was further compared amongst all three sets.

Total cell lysate
To obtain the total cell protein, a fully confluent T25 flask (~10^6) cells was trypsinized and the cell pellet was washed with ice cold 1X PBS once. The pellet was resuspended in 0.5ml protein extraction buffer (20mM HEPES Buffer, 20 % glycerol, 500 mM NaCl, 1.5mM MgCl2, 0.2mM EDTA, 0.1% Triton-X 100, 1mM PMSF, 1mM DTT) and incubated in ice for 1 hr with intermittent tapping at an interval of 10 min. The cells were then centrifuged at 10,000 rpm for 18 mins at 4°C. From the obtained 500µl of supernatant, 50µl was used for protein estimation using Bradford assay and remaining was immediately stored at -80°C with 5µl protease inhibitor.

Catalase assay
To study the possible effect vitamin D deficiency on mitochondrial metabolism in terms of ROS levels, the catalase assay was performed and assuming the catalase activity of set1 as 100%, the change in its activity was compared between all three sets. To begin with this assay, firstly the total cell lysate was prepared as per standard protocol. The assay buffer contained 2mM Hydrogen Peroxide in 67mM of phosphate buffer (pH 7.0), 30µg of protein was added to 3 ml of assay buffer. The decrease in absorbance was measured for 0 to 10 min at 240nm. The amount of H_2O_2 reduced was estimated by using a molar extinction coefficient of 43.1 M^-1 cm^-1 at 240 nm for H_2O_2.

RESULTS AND DISCUSSIONS

Cell gained 60% viability at 10^-9 M concentration of vitamin D
MTT assay was done to examine the cyto-protective effect of vitamin D on L6 cell lines. This assay revealed that L6 cells regained their viability by 60% at 10^-9 M concentration of vitamin D. Hence, this concentration of vitamin D was chosen as its optimum concentration to be used for all the further assays. As serum is source of vitamin D hence due to low serum concentration (2%), along with all other growth factors the vitamin D level is also low but as the cells are supplemented with optimum concentration of vitamin D, the viability of cells is improved. Prior studies have also reported cyto-protective effect of vitamin D in which 1,25 (OH)_{2}D_{3} induced expression of A20 anti-apoptotic protein in cytokine-treated human pancreatic islets, preventing cell death and also counteracted cytokine-induced Fas (death ligand) expression. In skin cells also, vitamin D has proven to protect from UV-induced skin damage.
Vitamin D supplementation helped in Calcium and glucose uptake by L6 cells simultaneously

Vitamin D is known to regulate intracellular calcium levels via two ways. Firstly by regulating calcium channels present in intestine, kidney, and pancreatic β cells etc. Secondly, it also regulate the level of calcium binding proteins known as calbindins and maintains the calcium level inside the cell.\(^{27}\) In our study also, effect of vitamin D on Calcium uptake was studied in L6 cells. As calcium is one of the components of the serum. Hence, it was observed that at a given time, media with 10% serum (Set1) had the maximum concentration of calcium as compared to 2% serum containing media (Set2). Treatment of L6 cells grown in 2% serum and \(10^{-9}\) M Vitamin D for 24hrs and 48hrs showed increased consumption of calcium. Thus, there was a decrease in calcium level in the media at 24 hrs and 48 hrs by 0.102 µg and 0.13 µg, respectively as, compared to negative control. Also, for each set, the calcium level was found to be decreasing with increase in time from 24 hr to 48 hrs. However, the results were inconclusive for 72hrs (Fig. 2). Thus, it may be suggested that calcium uptake of cells was improved with addition of vitamin D in the media, up to 48 hrs. We further tested whether there is any correlation of this increased calcium uptake with increased glucose consumption. The experiment was performed in all 3 sets. Glucose uptake was estimated by measuring remaining glucose levels in the media (Fig. 3). Glucose is the component of media but not serum and its uptake is also very much dependent on the concentration of other growth factors and vitamin D present in serum. Hence, as expected, since media with 10% serum (Set1) had optimum concentrations of all the required growth factors and nutrients, consumption of glucose was highest in this set and hence there was minimum concentration of glucose in the media of Set1 as compared to Set2 and 3. On the contrary, media with 2% serum (Set2) had all the growth factors but in the minimum concentration; hence, the consumption of glucose was low. However, when the optimized concentration of vitamin D (\(10^{-9}\) M) was supplemented to the media with 2% serum, consumption of glucose was increased. It was found that glucose consumption was increased approximately by 17%, 30% and 54 % at all different time points (24, 48 and 72hrs), respectively. Thus, we could see a similar effect of vitamin D on calcium consumption as well as on glucose uptake. Where there is more calcium uptake, there is increased glucose uptake too. This gave us the first indirect clue about the involvement of vitamin D in increased uptake of glucose via increased calcium uptake. The observation that calcium uptake is coupled with glucose uptake was in line with previous report of Lanner et al. (2006) where when the muscle cells isolated form limbs of adult mice were exposed to insulin, there was an increased calcium influx along with the increase in 2-deoxyglucose (2-DG) uptake. In the presence of agents who blocked calcium influx, glucose 2-DG uptake was also decreased pointing towards the coupling of calcium with glucose levels and role of calcium in insulin response.\(^{28}\)
Mitochondrial metabolism improved with vitamin D supplementation

Mitochondrial defects are proven to be associated with the pathogenesis of diabetes and vitamin D deficiency is also proposed to affect the mitochondrial activity. Hence, in order to understand if the vitamin D deficiency affects the process of glucose uptake by affecting the mitochondrial metabolism, further assays were performed in order to check the mitochondrial metabolic activity in case of vitamin D deficiency. For this, NADH oxidase and catalase assays were performed in all three set of experiments as these are the most common and direct tests to study the mitochondrial health.

Vitamin D supplementation improved NADH oxidase activity

To determine the activity of NADH oxidase, decrease of NADH concentration was observed in all the three set of experiments at 340 nm. The change in OD at 340 nm for 8 min was used for determination of NADH oxidase activity. As a result, it was observed that enzymatic activity was highest in cells growing in Set1 (as a reference, enzyme activity considered as 100%) and activity was least in cells growing in Set 2 (reduced by 74.4%). As expected, the NADH oxidase activity was observed to be improved by 24.2% when 2% serum containing media was supplemented with $10^{-9}$ M vitamin D (Table 1).

Table 1
Effect of vitamin D supplementation on NADH oxidase activity

<table>
<thead>
<tr>
<th>Experimental Sets</th>
<th>NADH oxidase activity (Mcm/min)</th>
<th>% NADH oxidase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Set1 (DMEM + 10% serum)</td>
<td>0.1688 ± 0.0012</td>
<td>100</td>
</tr>
<tr>
<td>Set2 (DMEM + 2% serum)</td>
<td>0.0410 ± 0.0024</td>
<td>25.6 ± 3.89</td>
</tr>
<tr>
<td>Set3 (DMEM + 2% serum + $10^{-9}$ M Vitamin D)</td>
<td>0.089±0.006</td>
<td>49.8 ± 1.24</td>
</tr>
</tbody>
</table>

NADH oxidase activity is expressed as Mean ± SEM
Effect of vitamin D supplementation on NADH oxidase activity (in %). Set1= DMEM + 10% serum, Set2= DMEM + 2% serum, Set3= DMEM + 2% serum+ 10^-9 M Vitamin D

As observed in the above graph, when cells were growing in media (Set2), NADH oxidase activity was reduced to 25% as compared to the activity in Set1. But when the media of these cells were supplemented with 10^-9 M vitamin D, NADH oxidase activity was regained to about 50% of the positive control. Though the supplementation of vitamin D did not help in full recovery of NADH oxidase activity, but the experiment suggested that vitamin D supplementation may improve the mitochondrial metabolic activity. Calcium has been long reported to have an important effect on mitochondrial activity. In its micromolar range, it has shown to modulate important enzymes participating in Kreb’s cycle; pyruvate, isocitrate, and α-ketoglutarate dehydrogenase also has shown to increase ATP synthesis in the mitochondria. And as vitamin D increases calcium uptake, it can also indirectly have an effect on mitochondrial metabolism. Our observations in L6 cells corroborate with previous findings where cholecalciferol therapy is reported to improve mitochondrial oxidative function in patients with severe vitamin D deficiency and skeletal muscle fatigue, they showed improved mitochondrial oxidative function.

Vitamin D supplementation improved Catalase activity
For estimation of Catalase activity, reduction of H2O2 was monitored at 240 nm and per min change in absorbance was used for calculation of enzymatic activity. The values were converted to percentage activity by considering the % activity of positive control to be 100% as reference (Table 2).

Table 2
Effect of vitamin D supplementation on catalase activity

<table>
<thead>
<tr>
<th>Experimental sets</th>
<th>Catalase activity (Mcm/min)</th>
<th>% Catalase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Set1 (DMEM + 10% serum)</td>
<td>0.101±0.011</td>
<td>100</td>
</tr>
<tr>
<td>Set2 (DMEM + 2% serum)</td>
<td>0.005±0.0002</td>
<td>5.77±0.425</td>
</tr>
<tr>
<td>Set3 (DMEM+ 2% serum+10^-9 M Vitamin D)</td>
<td>0.024±0.002</td>
<td>24.22±4.6</td>
</tr>
</tbody>
</table>

Catalase activity is expressed as Mean±SEM

Effect of vitamin D supplementation on catalase activity (in %). Set1= DMEM + 10% serum, Set2= DMEM + 2% serum, Set3= DMEM + 2% serum + 10^-9 M Vitamin D
As per the observation, when the cells were grown in 2% serum containing media, the catalase activity was found to be decreased to 5% as compared to the activity of the cells growing in 10% serum containing media. However, the supplementation of 10⁻⁹ M vitamin D to these cells, showed the considerable improvement in the activity of catalase and the activity was regained to about 25% of the positive control. Mitochondria continuously produce reactive oxygen species (ROS) as a result of constant metabolism of oxygen through electron-respiratory chain. Excessive accumulation of ROS is taken care by scavenging enzymes including catalase. In our study, since the NADH oxidase activity was reduced in mitochondrial fraction of cells grown in media with 2% serum, it is expected that the byproduct of electron-respiratory chain i.e. the ROS production will be decreased and hence, catalase activity is also expected to decrease as seen in the above graph.

CONCLUSION

Treatment of L6 cell lines (rat skeletal cells) with 10⁻⁹ M calcitriol showed an increase in calcium and glucose uptake as compared to control suggesting that vitamin D can positively regulate glucose uptake via increased calcium uptake. Vitamin D also increased mitochondrial metabolic activity as shown by increase activity of NADH oxidase and catalase enzyme in set of cells supplemented with vitamin D. These results may propose the anti-diabetic function of vitamin D via possibly improving the mitochondrial metabolism. As per our study, the proposed link between vitamin D and mitochondrial metabolic activity may allow for better understanding of mechanism of action of vitamin D and hence, can be further used as a therapeutic molecule in prevention and/or management of diabetes. Prospective clinical studies on vitamin D are required to firmly establish the role of vitamin D in the prevention and management of diabetes.

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CONFLICT OF INTEREST

Conflict of interest declare none.

REFERENCES


