

Neuroprotective role of vitamin D in primary neuronal cortical culture

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ABSTRACT

Background: A role of Vitamin D in brain development and function has been gaining support over the last decade. There are compelling pieces of evidence that suggest vitamin D may have a neuroprotective role. The administration of vitamin D or its metabolites has been shown to reduce neurological injury and/or neurotoxicity in a variety of animal systems. The detail biochemical mechanism mediating neurons, to its ability to withstand greater oxidative stress in the presence of Vitamin D is unclear. This study was undertaken to study the biochemical effect of treatments of primary cortical neuronal cultures, with the active form of vitamin D ($1,25(\text{OH})_2\text{D}_3$), against the induced oxidative stress.

Methods: Primary neuronal cultures from cerebral cortex were set up from neonatal (from 6 to 7 days old) Wistar Rat's brain. Different doses of $[1,25(\text{OH})_2\text{D}_3]$, ranges from 0 to $1 \mu\text{g/ml}$, was added to the culture medium and the cells were cultured in its presence for 24 h to 120 h. The effect of induced extracellular oxidative stress was measured by subjecting these cultured cells with $0.5 \text{ mM H}_2\text{O}_2$ for 2 h, prior to collection of condition medium and the cell pellet for biochemical assay. The control and H_2O_2 treated cultures were maintained in similar culture conditions, for similar periods of time without any $[1,25(\text{OH})_2\text{D}_3]$ treatments.

Result: The optimum concentration of $[1,25(\text{OH})_2\text{D}_3]$ for treatment of primary cortical neuronal cultures was found to be $0.25 \mu\text{g/ml}$ by Trypan exclusion assay and MTT assay. Pre-treatments of cultured neuronal cells with $0.25 \mu\text{g/ml}$ of $[1,25(\text{OH})_2\text{D}_3]$ caused significantly increased levels of reduced glutathione, accompanied by a similar increase in the enzyme levels of GST, to neutralize the induced oxidative stress by H_2O_2 . The level of Lipid peroxidation was significantly higher in the cells treated with H_2O_2 alone, but it was completely reversed in the neuronal cultures pre-treated with $[1,25(\text{OH})_2\text{D}_3]$. The levels of Catalase enzyme also significantly reduced (≥ 0.05) in the $[1,25(\text{OH})_2\text{D}_3]$ pre-treated neuronal cultures.

Conclusion: We concluded that the systemic treatment of primary neuronal cultures with $[1,25(\text{OH})_2\text{D}_3]$ gave better protection to neurons against the induced oxidative stress, as shown by quantitative measurements of various biomarkers of oxidative stress. This study also suggested that Vitamin D is vital for the growth, survival, and proliferation of the neurons and hence it has a potential therapeutic role against various neurodegenerative diseases.

1. Introduction

Oxidative stress, often described as the disruption of the balance between the pro-oxidant and anti-oxidants due to the excessive accumulation of reactive oxygen species (ROS). The central nervous system is highly vulnerable to these ROS, due to its high energy demand and its high metabolic rate, in contrast to its low levels of the antioxidant defense system and its reduced capacity of cellular regeneration. In case of Parkinson's disease (PD), Alzheimer's Disease (AD) and Amyotrophic Lateral Sclerosis (ALS), several indices of ROS damage have been reported within the specific brain regions that undergo selective neurodegeneration. For example, the markers of Lipid peroxidation including 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA), have been identified in the cortex and hippocampus of patients with an AD, the substantia nigra of patients with PD and in spinal fluid from patients with ALS [1–4]. Protein nitration, a marker of protein oxidation, has been demonstrated to be elevated in the hippocampus and neocortex of individuals with an AD, in Lewy bodies in case of PD and within motor neurons in ALS [5]. In light of the depilating effect of ROS in the pathophysiology of a brain, there is an urgent need to explore the

therapeutic potential of various antioxidants. One such antioxidant which is discovered recently is Vitamin D. The role of Vitamin D in the brain development and function of neurons was suggested when the first evidence of 1α -hydroxylase an enzyme responsible of an active form of Vitamin D and receptors for vitamin D was found to be present in the brain [6]. The specific mechanisms that mediate the neuroprotective effect of vitamin D are still unclear; however, vitamin D may act in many pathways including antioxidant pathways, neuronal calcium regulation, immunomodulation and glutamatergic systems [7–10]. A study on the biologically active vitamin D3 metabolite, Calcitriol, added to the rat primary dopamine neurons, showed a dose response increase in numbers of these neurons due to the upregulations of glial derived neurotrophic factor (GDNF) [11,12]. In the present study, the antioxidant potency of $1,25(\text{OH})_2\text{D}_3$ had been studied in detail using an *in-vitro* model of neuronal damage caused by induced oxidative stress by exposure to hydrogen peroxide. The therapeutic potential of active Vitamin D3 metabolite, on the primary cortical neurons, was investigated in detail by quantitative measurements of major antioxidants enzyme systems.

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2. Method

2.1. Setting up primary cortical cell culture

Five neonatal Wister Albino rats of about 6 to 7 days old (with average weight of 20 g) were used for this study. The rats were kept at a facility of King Saud university research Centre under the strict guidelines provided by the Experimental Animal Laboratory and approved by the animal care and use committee at the College of Applied Medical Sciences at King Saud University. All procedures dealing with animals were followed in accordance with the standard ethically approved protocol.

The brains of the rats were removed immediately after being anesthetized and decapitated in a sterile condition. The meninges were removed and the midbrain (cortex) was isolated and rinsed with sterile Phosphate Buffer Saline (PBS) pH 7.4, and finely minced into small pieces. The minced tissue was then incubated with Papain solution (2 mg/ml) for 20 min at 37 °C as described previously [13]. The cell suspension was filtered through the cell strainer and cell debris and big tissues were discarded. The cell suspension was centrifuged for 300 × g for 10 min, the supernatant was discarded and the cell pellet was suspended in plating medium DMEM F12 with glutamine (Sigma Aldrich) + 10%FCS + 100 units/ml penicillin + 0.1 mg/ml of streptomycin. The cell pellet was washed twice in this medium and after that, the cells were counted using Bio-Rad automated cell counter. The cells were plated onto 12well cell culture dishes (Millipore Company) with a plating density of 5×10^6 cells/ml.

2.2. Treatment of cortical cells in culture

Different doses of [1,25(OH)₂D₃] that is (0.25 µg/ml, 0.5 µg/ml, 0.75 µg/ml and 1 µg/ml) was added to the cells in cultured medium just after plating. The cells were treated with different doses of [1,25(OH)₂D₃] in triplicate and cultured for up to 120 h. At the end of each set of incubation periods, the cells were briefly induced oxidative stress, by treating with 0.5 mM H₂O₂ for 2 h. The control and H₂O₂ treated cultures were grown without any treatments of [1,25(OH)₂D₃]. All the cultures were maintained in DMEM F12 medium with glutamine (Sigma Aldrich) + 10%FCS + 100 units/ml penicillin + 0.1 mg/ml of streptomycin for the selected time periods at 37 °C in an atmosphere of 95% air and 5% carbon dioxide.

2.2.1. Trypan blue exclusion assay

The cells were grown in 12 well plates, with different treatments and the cell viability is determined at different time points that is 24 h, 48 h, 72 h and 120 h by trypan blue exclusion assay. Triplicate wells of a viable cell were counted on an automated counter (BIO-RAD TC10).

2.2.2. MTT assay

For the MTT assay the MTT cell growth Kit from Millipore (CT02) was employed. The cells were plated on a 96well plates with a cell density of 1×10^4 /well and treated with various concentration of [1,25(OH)₂D₃] that contains 100 µl of DMEM F12 medium + 100 units of penicillin and streptomycin. Wells with culture medium without FBS was taken as negative control. The cells were grown for 24 h, after that they were treated with 0.5 mM H₂O₂ for 2 h. The control cells were plated in the same conditions without any treatments. After this 10 µl of MTT solution (2-5dipheyltetra sodium bromide in PBS) was added, and incubated in its presence for 4 h. 100 µl of isopropanol in 0.04 N HCl was added to each well. The absorbance was read at 530 nm within one hour. Each sample was done in triplicate wells.

2.2.3. Reduced glutathione assay

The estimation was carried out by the method of Beutler et al. [14]. 1 ml of cell homogenate and 1 ml of conditioned medium was used for this assay. 1.5 ml of double distilled water was added to the tissue

homogenate, and conditioned medium, and treated with 0.6 ml of precipitating reagent (containing 1.67 g of glacial metaphosphoric acid, 0.2 g of EDTA and 30.0 g of NaCl made up to 100 ml with double distilled water). The above reaction mixture was centrifuged at 1200 × g for 10 min. To 0.3 ml of supernatant, 2 ml of Na₂HPO₄ (0.3 M) and 0.25 ml of 5,5' dithio-bis-2-nitrobenzoic acid (DNTB)(0.4% in 1% sodium citrate) were added, and volume was made up to 3 ml with DDW. OD was read at 412 nm against blank. Values were expressed as µg of reduced glutathione/No of cells present.

2.2.4. Lipid peroxidation assay

Lipid peroxidation was calculated using the method of Garcia et al. [15], using the TBARS (Thiobarbiturate reactive substances) assay. 1 ml of Brain homogenate was incubated in a metabolic shaker at 37 °C for one hour. 1.5 ml of 20% TCA was added, and centrifuged at 600 × g for 10 min. 1 ml of freshly prepared TBA (0.67%) was added to 1 ml of the supernatant, and the reaction mixture was heated in a boiling water bath for 10 min. Absorbance was read at 535 using a reagent blank. Values were expressed as expressed as mMoles of Malondialdehyde formed hour/ No of cells.

2.2.5. Catalase assay

Catalase activity was estimated in the cell homogenate, as well as a conditioned medium by the method of Aebie, 1984 [16]. The reaction mixture in a total volume of 3 ml contained 0.4 M sodium phosphate buffer (pH 7.2). The reaction was started by adding 1.2 ml of H₂O₂ and reading the change in absorbance at 240 nm for 2 min. One unit of CAT activity was defined as micromole of H₂O₂ decomposed per minute using the molar coefficient of H₂O₂ ($43.6 \text{ M}^{-1} \text{C}^{-1}$).

2.2.6. Glutathione S transferase assay

The activity of Glutathione S-transferase was assayed in a reaction mixture containing 100 mM phosphate buffer, pH 6.5, 1 mM 1-Chloro-2,4-dinitrobenzene (CDNB) and 1 mM reduced GSH. The reaction was initiated by adding 10 µl of Cell lysate and formation of S-(2, 4-dinitrophenyl) glutathione (DNP-GSH) was measured spectrophotometrically as Units per minute per 5×10^6 cells [17].

2.2.7. Statistical analysis

All data are expressed as the ± Standard deviation (SD) of at least three independent replicate experiments. Students' *t*-test was performed to assess the difference between the control and treated groups. Comparison between the Control, H₂O₂ treated and vitamin D treated groups of cells were made using two-way analysis of variance (ANOVA) and Tukey's *post hoc* test. Values of ≤ 0.05 were considered to be significant.

3. Results

3.1. Effect of vitamin D treatments on the cell viability

The cortical neurons were cultured on 96 well plates with a cell density of 1×10^4 cells/well for 24 h, 48 h and 72 h in the presence and absence of varying doses of [1,25(OH)₂D₃] ranges from 0 to 1 µg/ml. After each incubation period, the cells were subjected to oxidative stress, by adding 0.5 mM of H₂O₂ to the culture medium for 2 h. Cell viability was measured by MTT assay as well as trypan blue exclusion assay by counting them on the automated counter as shown in Fig. 1. Both of these data showed that the optimum treatment dose was 0.25 µg/ml. Hence this dosage was used subsequently in the latter experiments. MTT assay showed that the treatment with 0.25 µg/ml of [1,25(OH)₂D₃] resulted in the greater protection of the neurons against the induced oxidative stress by H₂O₂ when compared to the H₂O₂ treated alone samples. These results were further supported by Trypan blue exclusion assay in which the total viability of the cells was counted after 24 h, 48 h and 72 h of the treatment with the 0.25 µg/ml and

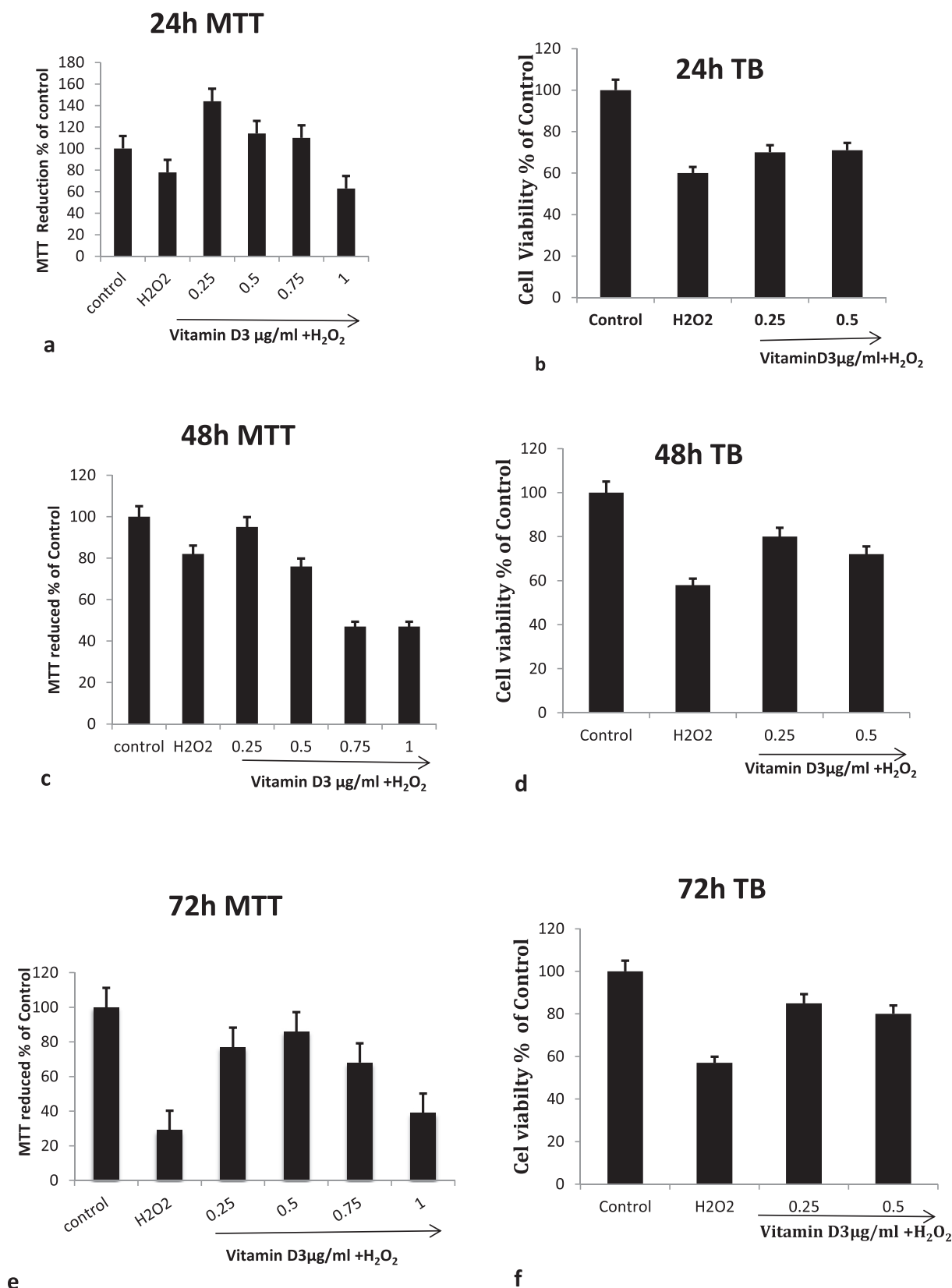
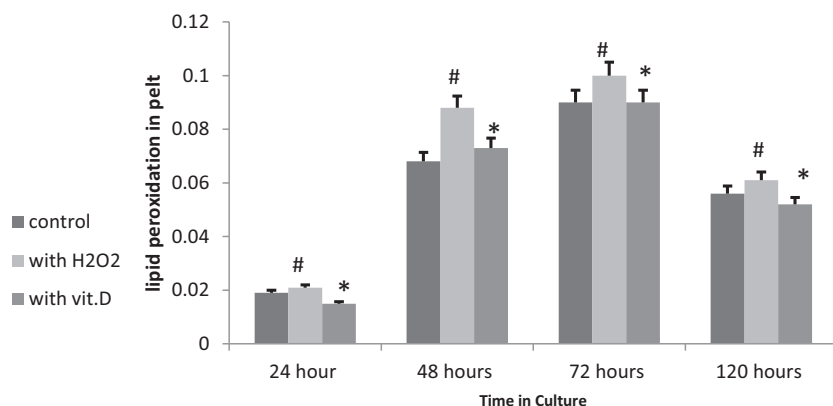


Fig. 1. Effect of Different doses of Vitamin D treatments on the viability of primary cortical neuronal cells after induced oxidative stress by 0.5mMH₂O₂ for 2 h after growing them in the presence and absence of [1,25(OH)₂D₃] for 24 h, 48 h and 72 h. The cell viability was measured either by MTT reduction for 24 h, 48 h and 72 h (a, c, e) or by Trypan blue exclusion assay (b, d, f). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Estimation of lipid peroxidation in Cell pellet



0.5 µg/ml of [1,25(OH)₂D₃]. The cells which were treated with H₂O₂ alone lost about 40% of cell viability, as compared to the control cells. However, when the cells were pretreated with [1,25(OH)₂D₃], they showed improved cell viability.

These results suggested that systemic pre-treatments with [1,25(OH)₂D₃] to the neuronal cultures protected them, against induced oxidative stress apoptosis.

3.1.1. Effect of vitamin D treatments on lipid peroxidation levels

Cells under oxidative stress have a greater amount of lipid peroxidation, which can be measured by TBRAS assay, which detects the amount of Malondialdehyde (MDA) formed in the cell pellet. Our results showed that the Cells which were treated with the H₂O₂ alone, showed significantly increased amount of MDA. However the neuronal cells cultured for 24 h to 120 h, in the presence of [1,25(OH)₂D₃] showed greater tolerance to the H₂O₂ induced oxidative stress, as exhibited by significantly reduced MDA formation (Fig. 2).

3.1.2. Effect of vitamin D on reduced glutathione levels

In order to elucidate, the state of antioxidants enzyme mechanisms, through which [1,25(OH)₂D₃] might confers neuroprotection. We measured the total amount of reduced GSH both, in the cell pellet as well as the conditioned medium, after various time points. Reduced GSH present in the cell pellet as well as in the conditioned medium at 24 h was elevated when compared to the H₂O₂ treated alone and control Cells. The same elevation of reduced GSH was observed in the cell cultures that were pretreated with [1,25(OH)₂D₃] (0.25 µg/ml) for 48 h, 72 h and 120 h. While the control and H₂O₂ treated Cells had reduced GSH levels (Fig. 3). These results further confirmed earlier studies, that one of the mechanisms through, which [1,25(OH)₂D₃] protects the neurons was by upregulation of antioxidant enzyme defense systems [18].

3.1.3. Effect of vitamin D on catalase enzyme

Catalase is an active enzyme which can neutralize the effect of H₂O₂ by converting them to water. This enzyme is the main antioxidant enzyme present in the cells which fight against the oxidative stress by rapidly breaking down H₂O₂ produced into the water, thus alleviating its harmful effects. The level of catalase enzyme was measured in the [1,25(OH)₂D₃] treated cultures for 24 h, 48 h, 72 h and 120 h. We found that the Catalase enzyme activity in the cell pellet of the [1,25(OH)₂D₃] treated cell cultures, was found to be reduced significantly (Fig. 4). The same observation was found in the cell pellet of the cultures derived from 48 h, 72 h and 120 h of treatment with [1,25(OH)₂D₃], and exposed to H₂O₂ induced oxidative stress, thus indicating that [1,25(OH)₂D₃] treatments protected the neurons from induced oxidative stress, as revealed by low levels of catalase enzyme being present.

Fig. 2. Estimation of Lipid peroxidation in the cell pellet after culturing the primary cortical neuron cells in the presence and absence of 0.25 µg/ml of [1,25(OH)₂D₃] for 24 h, 48 h, 72 h and 120 h, after that they were induced oxidative stress by exposure to 0.5 mM H₂O₂ for 2 h. Cell pellet was isolated and of Lipid peroxidation was estimated as described in Materials and Methods. (*) represent the values of [1,25(OH)₂D₃] treated samples which were significantly different (≥0.05) from the H₂O₂ treated alone samples. (#) represent the significant different (≥0.05) values of H₂O₂ treated samples compared to the control samples.

The neuronal cultures exposed to H₂O₂, without any pretreatments of [1,25(OH)₂D₃] showed increased amounts of catalase enzyme, (Fig. 3) indicating that the neurons were under direct exposure to oxidative stress by hydrogen peroxide with no protection, so increased amount of catalase enzyme released to neutralize its effect.

3.2. Effect of vitamin D on glutathione S transferase enzyme activity

The Glutathione S transferase (GST) constitutes a family of cytosolic and membrane-associated microsomal proteins that are involved in the detoxification of electrophilic xenobiotics. Immunohistochemical studies and spectrophotometric enzyme assay suggested the presence of GST enzyme activity in Purkinje cells throughout the cerebellar cortex in the rat's brain [17]. The presence of GST enzyme in the brain suggests that it gives protection to the neurons against exogenous and endogenous neurotoxic compounds. The amount of GST enzyme was significantly lower in the neuronal culture exposed to H₂O₂ induced oxidative stress at 24 h, 48 h and 72 h in culture (Fig. 4). However, the presence of [1,25(OH)₂D₃] in the neuronal culture significantly reduced this oxidative stress by upregulating the GST enzyme levels, clearly indicating the neuroprotective potential of [1,25(OH)₂D₃].

4. Discussion

Vitamin D deficiency was suggested to trigger premature aging [19], enlargement of the lateral ventricle, reduction in NGF protein content, reduction in expression of a number of genes involved in neuronal structure, disruption of brain development and following persistent changes in the adult brain of animals [7]. Limited but collateral findings in human studies were also reported. Low levels of the plasma 25hydroxyvitamin D (25OHD) were suggested to be associated with mood disorder, dementia, mild cognitive impairment, Alzheimer's disease and autism [20–24]. These studies led us to use Vitamin D in our *in-vitro* studies and explore its therapeutic potential in detail.

Initial studies using MTT assay and trypan blue exclusion viability assays suggested that 0.25 µg/ml was the optimum dosage while higher doses are toxic to the cells. So this dosage was used for the treatments to the cortical neurons in culture. Similar dosage of [1,25(OH)₂D₃] was used in other *in-vitro* studies [8].

Cells under oxidative stress release free radicals which cause the peroxidation of the polyunsaturated fatty acids in the form of MDA as the final product. Treatment with H₂O₂ alone resulted in significantly high levels of MDA, while the cells that were pretreated with [1,25(OH)₂D₃] and then exposed to induced oxidative stress by H₂O₂ were completely protected from the excess generation of free radicals as shown by the significantly low levels of MDA. The protection from oxidative stress seemed to be more and more significant in the cultures

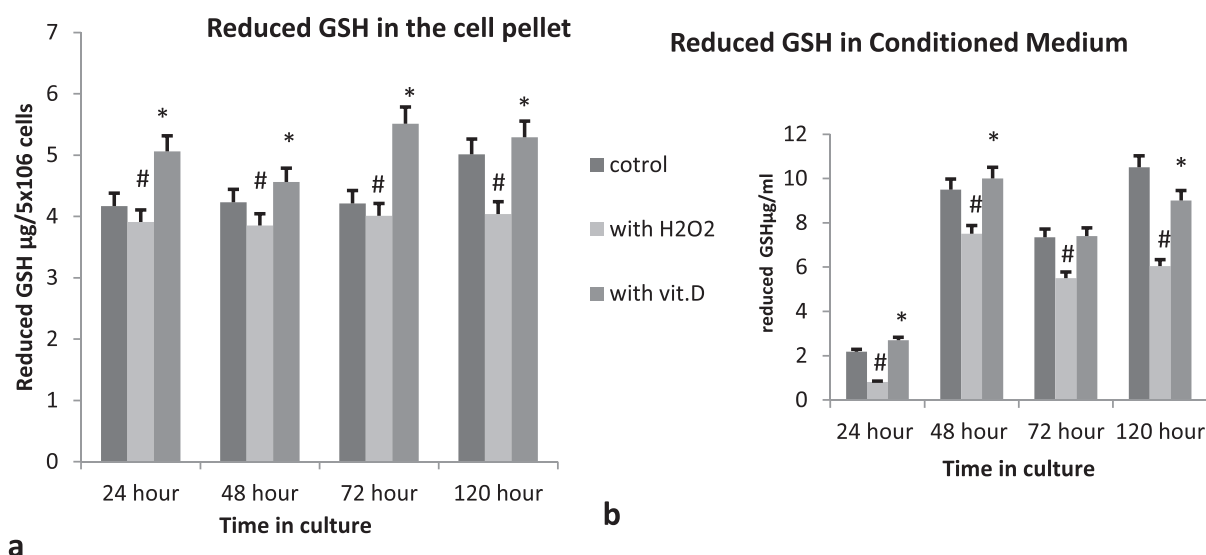


Fig. 3. Estimation of reduced Glutathione from Cell pellet and conditioned medium derived from primary cortical neuron cultures grown in the presence and absence of 0.25 µg/ml, [1,25(OH)₂D₃] for 24 h, 48 h, 72 h and 120 h, after that they were exposed to 0.5 mM of H₂O₂ for 2 h. Conditioned medium and cell pellet was separated by centrifugation. Reduced GSH was estimated in both conditioned medium and the cell pellet as described in the methods section. (*) represent the values of Vitamin D treated groups which are significantly (≥ 0.05) different from the H₂O₂ treated alone samples. Each reading is an average of three readings from three independent sets of experiments. (#) represent the H₂O₂ treated samples significantly different to the control groups.

that were exposed to H₂O₂ after 48 h, 72 h and 120 h of pretreatment with Vitamin D.

It has been reported that treatment with [1,25(OH)₂D₃] can upregulate glycine –cysteine ligase catalytic subunit and Glutathione reductase and increased cellular GSH formation in cultured Monocytes [18]. Studies showed that vitamin D₃ exerts its protecting effects against free radicals generation by reactive species of oxygen and nitric oxide by inhibiting the synthesis of inducible nitric oxide synthase, and by regulating the activity of the gamma-glutamyl transpeptidase, which is a key enzyme involved in the metabolism of glutathione [25]. In the present studies, the reduced GSH was shown to be significantly upregulated with the pretreatments of neuronal cultures with [1,25(OH)₂D₃] after 24 h, 48 h, 72 h and 120 h. GST is one of the other antioxidant enzymes which plays a critical role in combating oxidative stress by catalyzing the conjugation of glutathione and various free radicals formed by toxins, thus helping in their elimination and maintaining the redox state of cells. In our study, neuronal cells treated with H₂O₂ alone caused significantly reduced levels of GST enzyme activity

which agrees with our earlier finding of less amount of reduced GSH in those cultures. However with the pretreatment of these cultures with [1,25(OH)₂D₃] for 24 h, 48 h and 120 h substantially improved the GST enzyme activity as well as increased amount of Reduced Glutathione. These results clearly indicated the direct relationship of reduced GSH and GST enzyme levels being upregulated with the pretreatment of [1,25(OH)₂D₃] and their combined effect in alleviating the oxidative stress induced by H₂O₂.

Catalase enzyme prevents excessive accumulation of the strongly oxidizing agent H₂O₂ which otherwise can do damage to the cells. Because of this preventive effect of catalase, important cellular processes which generate H₂O₂ as a by-product can proceed safely. Biochemical analysis of catalase has shown that it binds endogenously to 24,25(OH)₂D₃ [26]. In our results, the catalase enzyme activity was significantly reduced in the presence of [1,25(OH)₂D₃], while the cultures treated with hydrogen peroxide alone showed a marked increased level of catalase enzyme in the cell pellet. This finding again showed the antioxidant nature of Vitamin D₃ metabolite which is protecting

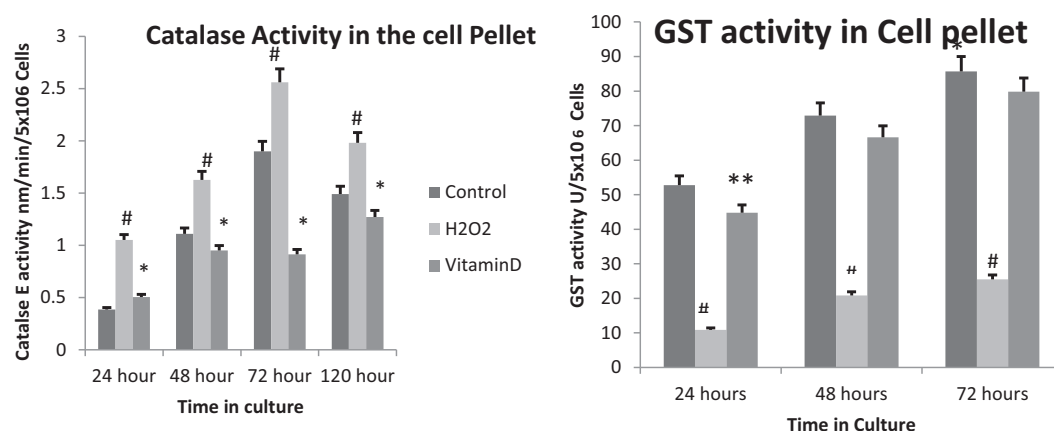


Fig. 4. Estimation of Catalase enzyme and GST enzyme activity as measured in the cell pellet derived from primary cortical neurons cultures grown in the presence and absence of 0.25 µg/ml of [1,25(OH)₂D₃] for 24 h, 48 h, 72 h and 120 h, followed by exposure to 0.5 mM of H₂O₂ for 2 h. (*) represents the *P* value as [1,25(OH)₂D₃] treated samples significantly different (≥ 0.05) from the H₂O₂ treated samples. (#) represent the H₂O₂ treated samples compared to the control samples as significant.

neuronal cells from the harmful effect of H_2O_2 . In the absence of Vitamin D3 treatments to the neuronal cells, H_2O_2 caused a significant upregulations of catalase enzyme in order to neutralize its damaging effect to the neurons.

5. Conclusion

Oxidative stress is the main contributor to various neurodegenerating diseases, and the neuroprotective role of Vitamin D has been gaining support over the last decade. In this study, we looked at the various metabolic markers for oxidative stress such as Lipid peroxidation (levels of MDA), reduced glutathione levels and GST enzyme levels as well as catalase enzyme levels, with the long-term treatment of neuronal cultures with vitamin D. The data presented here further emphasized the therapeutic role of vitamin D3 in alleviating oxidative stress and its potential role in neuroprotection.

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