



Research Article

Vitamin D Treatment Reverses the Induced Oxidative Stress Damage to DNA

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Abstract

Background and Objective: The aim of the current study was to investigate in detail the effect of the active metabolite of vitamin D3 [1, 25 (OH)₂ D3] in ameliorating the induced oxidative damage to DNA. **Materials and Methods:** Primary cortical neuron cultures from one week old Wister rats were set up in sterile conditions. The neuron cultures were maintained for up to 72 h in culture in the presence of varying doses of vitamin D. Cells were exposed to (0.5 mM H₂O₂) for 2 h prior to collection of condition medium and cell pellet for Biochemical Assays. Control and H₂O₂ treated cultures were maintained without any treatment with vitamin D. **Results:** Pre-treatment with 0.25 µg mL⁻¹ for 24 and 48 h significantly reduced the oxidative stress. 8-hydroxydeoxyguanosine a ubiquitous marker of oxidative stress had also shown to be significantly reduced. The DNA damage marker PolyUB of histones was observed in the neuron treated with H₂O₂ only. **Conclusion:** This study revealed that oxidation of DNA by hydrogen peroxide caused extensive DNA damage, resulting in polyubiquitination of histones. The pre-treatment with vitamin D3 however completely reversed the DNA damage cascade induced by hydrogen peroxide and protected the DNA.

Key words: Oxidative stress, vitamin D, neurodegeneration, DNA damage, polyubiquitin (polyUb), monoubiquitination

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Oxidative stress has been associated with neuronal cell death and proved to be the main cause of various neurodegenerative diseases^{1,2}. Oxygen is required to meet the energy demand of the nervous system but the by-product of this metabolism results in Reactive Oxygen Species (ROS). The major members of the ROS family include superoxide (O_2^-) hydrogen peroxide (H_2O_2) and singlet oxygen (1O_2)³. The ROS are highly unstable and reactive and readily reacts with the surrounding molecules causing extensive damage and apoptosis. The damage caused to the DNA mainly in the form of oxidative DNA damage and cytogenetic damage contributes to the progression of the neurodegenerative process. The oxidative attack on DNA by ROS results in more than 20 oxidized bases adducts, the prominent among them is 8-hydroxyguanosine (8-OHG)⁴. This is the reason behind using 8-OHG used as a marker of DNA oxidation and damage as a quantitative biochemical assay. The integrity of DNA is greatly compromised by the presence of these ROS and cause single and double strands of DNA breaks and fragmentation. In response to DNA damage, the cell activates highly complex and conserved Kinase-based signaling response commonly termed as DNA damage response (DDR) to safeguard genomic stability⁵. The DDR constitutes an array of tightly regulated events, that include first of all detection of the damaged DNA, recruitment of a number of DNA repair factors and proteins and finally repairing the lesion. In the case of the DNA damage beyond repair, the cell signals the apoptotic machinery that results in the demise of the damaged cell. These very well defined and organized actions involve the transcriptional activation of a number of genes that govern the DDR. Recently the role of ubiquitination in orchestrating the DDR provided the dynamic cellular regulatory circuit and in the maintenance of genomic integrity and survival^{6,7}. Ubiquitin (Ub) is a 76 amino acid protein of ~8.5 kDa ubiquitously expressed in all cell. It can be found as a free form or covalently attached to a target protein. The massive accumulation of Ub at the site of DNA damage has been observed.

Mono and polyubiquitination of the core histones, linker histone H1 and histone variants have been reported. For example, in the events of DNA damage response, the E3 ligase RNF168 monoubiquitinates H2A at K13 or K15. Then, a K63-linked polyUb chain is added to the mono-ubiquitinated H2A. The above ubiquitination events have been shown to be involved in the DNA damage repair pathway. In the early steps of the DNA damage response, the E3 ligase RNF168 monoubiquitinates H2AK13/15. Then a K63 polyUb chain is

added on the H2AK13/15Ub. This serves as a signal to recruit the DNA repair proteins⁸: BRCA1 and 53BP1. Ubiquitinated H2B has also been implicated in the DNA damage response. The RNF20/RNF40 complex is recruited to DNA double-strand break sites where it catalyzes H2B monoubiquitination. Furthermore, depletion of RNF20 disrupts the recruitment of DNA repair proteins in both non-homologous ends joining and homologous recombination repair pathways⁹.

The ubiquitous presence of vitamin D receptor for its metabolite [1, 25 (OH)₂ D₃] opens up the possibility of its unique biological role as 'guardian of the genome' was unknown before. One of the suggested roles documented is that it acts as antioxidant and may prevent genetic changes by preventing DNA damages directly induced by free radical and ROS attack^{10,11}. In this study, the effect of vitamin D treatments on the cortical neurons in culture was explored and studied in detail, the effect on the integrity of DNA and genome stability.

MATERIALS AND METHODS

Setting up the primary cortical neuron culture: Four 1 week old Wister Albino rats 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. Cerebral cortex was separated from the front and hind lobe. The meninges were removed and the midbrain (cortex) was isolated in sterile conditions. Tissues were rinsed with sterile PBS and finely minced into small pieces. The minced tissue was then incubated with Papain solution (2 mg mL⁻¹) for 20 min at 37°C. The cell suspension was filtered through the cell strainer and cell debris and big tissues were discarded. The cell suspension was centrifuged for 300xg for 10 min, the supernatant was discarded and the cell pellet was suspended in plating medium DMEM F12 medium with glutamine (Sigma Aldrich)+10%FCS+100 units mL⁻¹ penicillin+0.1 mg mL⁻¹ of streptomycin. The cell pellet was washed twice in this medium and after that, the cells were counted using a Bio-Rad automated cell counter. The cells were plated onto 12 well cell culture dishes (Millipore company) with a plating density of 5×10^6 cells mL⁻¹.

Treatment of cortical cells in culture: Just after plating cells were treated with varying doses of the active metabolite of vitamin D (1, 25 (OH)₂ D₃ (0.25, 0.5 and 0.75 µg mL⁻¹) in triplicate and cultured in its presence for different time points from 24-120 h in culture. After that, the cells were induced oxidative stress by treating with 0.5 mM H₂O₂ for 2 h. The control and H₂O₂ treated cultures were grown without any vitamin D treatments.

All the cultures were maintained for the selected time periods at 37°C in an atmosphere of 95% air and 5% carbon dioxide. The conditioned medium was removed after each set of incubation and protease inhibitor (EDTA and phenylmethylsulfonyl fluoride (PMSF) added to the final concentration of .1 mM for 30 min at 4°C followed by freezing them at -80°C. The cell lysate was dissolved in PBS and protease inhibitor added as before and allowed to precipitate at 4°C in an excess of ice-cold acetone. Total protein of the samples was determined using the bicinchoninic acid (BCA) assay (Thermo Fisher Scientific USA). Equal amounts of proteins were resolved on 15% SDS-polyacrylamide gel electrophoresis (PAGE).

Western blotting: Monoclonal primary mouse antibodies (Ub and Tubulin) were purchased from Cell Signaling (United States). Secondary fluorescent mouse antibody was purchased from Li-COR Biosciences (United States). All chemicals and Western blot materials were purchased from Sigma. Whole-cell lysates of treated cells were loaded on a 15% SDS-PAGE and run for 1 h at 170 V. The protein gel was then placed on a nitrocellulose membrane. Bands were detected, in the near infrared region (700 nm), using Odyssey CLX Imaging System (LI-COR Biosciences, United States).

MTT cell proliferation assay: For MTT assay the MTT cell growth Kit from Millipore (CT02) was employed. The cells were plated on 96 well plates with a cell density of 1 × 10⁴/well and treated with varying concentration of 1, 25 (OH)₂ D₃ that contains 100 µL of DMEM F12 medium+100 units of penicillin and streptomycin. Wells with 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 (2-5 diphenyltetrazolium sodium bromide in PBS) was added and incubated in its presence for 4 h. After that 100 µL of isopropanol in 0.04 N HCl was added to each well. The absorbance was read at 490 nm within 1 h with a BIO-Rad (USA) microplate reader. Each

sample was done in triplicate wells. The cell growth curve was determined using the average absorbance at 490 nm from triplicate samples of three independent experiments.

DNA fragmentation and quantitation assay: The extent of DNA fragmentation was determined by the method described by Gercel-Taylor¹². The DNA precipitate was heated to 90°C for 10 min in 1 mL of 5% TCA and quantitative analysis was carried out by reaction with diphenylamine at room temperature for overnight. The O.D was measured at 600 nm against blank. The percentage of fragmentation was calculated as the ratio of fragmented DNA in the supernatant to the total intact DNA in cell pellet.

DNA extraction and electrophoresis: The effect of H₂O₂ on DNA fragmentation was examined by gel electrophoresis as described by Lee *et al.*¹³. The DNA samples were mixed with loading buffer and loaded onto a 1.8% agarose gel. The DNA was visualized by ethidium bromide staining and photographed.

DNA damage assay by OxiSelect™ oxidative DNA damage ELISA Kit ((8- OHdG quantitation): A competitive ELISA for 8-OHdG was performed using the ELISA kit (Cell Biolabs Inc., Life Sciences USA; Cat No; STA 322). Total genomic DNA was extracted using the DNA extraction kit by Millipore Life Sciences Research USA. The DNA was converted to single strand by incubating the samples at 95°C for 5 min and rapidly chilling on ice. The DNA was digested to nucleosides by incubating the denatured DNA with 20 units of nuclease P1 for 2 h followed by treatment of 10 units of alkaline phosphatase for 1 h at 37°C in a final concentration of 100 mM Tris pH 7.5. The reaction mixture was centrifuged for 5 min at 6000xg, the supernatant was used for 8-OHdG ELISA assay. About 0.5 mL of conditioned medium of all the samples was also used for DNA damage by 8-OHdG ELISA assay. The plates were read at 450 nm. The amount of 8-OHdG was determined for each sample from the standard curve.

Statistical analysis: All data were 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 0.01 were considered to be significant.

RESULTS

Effect of vitamin D on cell proliferation: To evaluate the effect different concentration of vitamin D on the proliferation rate of cortical neurons in culture for up to 72 h; $1, 25(\text{OH})_2 \text{D}_3$ was added at the beginning of culture at the time of plating. Cell proliferation was assayed by MTT as described in Methods section. Figure 1 showed that the optimum amount of $1, 25(\text{OH})_2 \text{D}_3$ that was $0.25 \mu\text{g mL}^{-1}$ accelerated the rate of proliferation as compared to the control and the cultures treated with $0.5 \mu\text{g mL}^{-1}$. This proliferation curve indicated that the addition of vitamin D helps in the proliferation and survival of the neurons.

DNA fragmentation assay: Oxidative damage to DNA results in chromosomal aberration, abnormalities in chromosome structure results in single and double strand breaks thus resulting in massive DNA fragmentation and apoptosis of the cells. Diphenyl assay is a very useful method for measuring apoptosis by determining the percentage of oligosomal-sized fragments¹⁴. Another advantage of this assay is that the percentage of DNA fragmentation is calculated for both adherent and suspended cells following treatment with the chemotherapeutics or any other agents. The results of this assay clearly showed that treatments of cells with H_2O_2 results in about 70% of the genomic DNA being disintegrated and fragmented (Fig. 2). The cells that were pretreated with vitamin D ($0.5 \mu\text{g mL}^{-1}$) for 24 h prior to the induced oxidative stress by H_2O_2 results significantly ($p < 0.001$) reversing the deleterious and damaging effect of H_2O_2 alone and completely protected the DNA. This study was further confirmed when the total genomic DNA from these samples was isolated and run on 1.8% agarose gel. The control samples have one high molecular weight distinctive band showing intact genomic DNA. The H_2O_2 treated samples, on the other hand, resulted in the severe oxidative damage to the DNA as shown in the gel as smearing of the whole genomic DNA (Fig. 3).

The cells that were pretreated with vitamin D for 24 h prior to inducing oxidative stress by H_2O_2 completely reversed the oxidative stress and their genomic DNA was protected from any degradation and fragmentation (Fig. 3 lane 4).

DNA damage by 8OHG assay: The guanine analog 8-hydroxyguanosine is an abundant base modification in mammalian DNA, the level of these 8-OHdG increases with an increase in oxidative stress. Increased levels of 8-OHdG are noticed to be higher in irradiated DNA and H_2O_2 treated DNA.

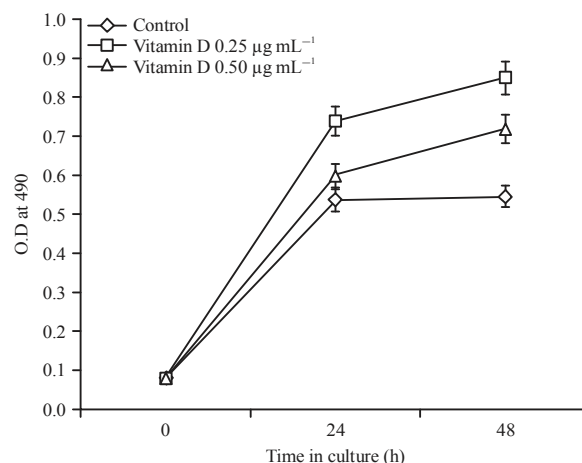


Fig. 1: Effect of treatment of vitamin D on the proliferation of the cortical neurons

Cortical region neurons were cultured on 96 well plates for 24, 48 and 72 h as described in Methods section. The MTT assay was performed and absorbance was measured at 490 nm. Here absorbance (490 nm) is directly proportional to cell viability

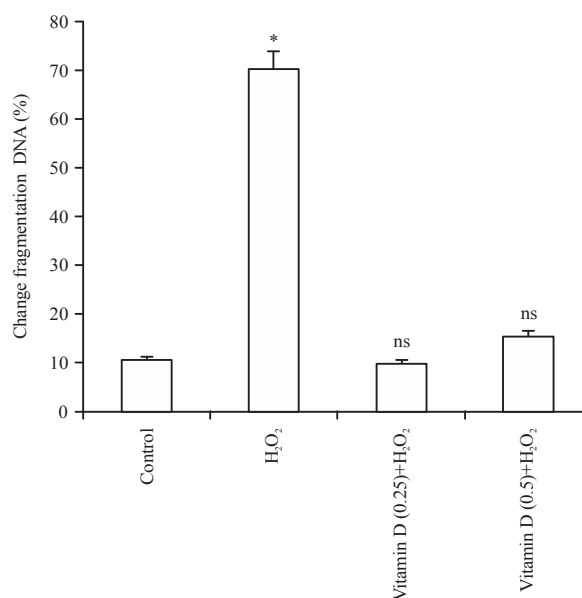


Fig. 2: Protective effect of vitamin D against H_2O_2 induced DNA damage

DNA fragmentation (%) was assessed by DPA assay in the control as well as Vitamin D treated (0.25 and $0.5 \mu\text{g mL}^{-1}$) groups as described in methods section, values are expressed as \pm SEM, $n = 6$ compared to control groups. Data between the groups were an analysis of variance (ANOVA) by Tukey's multiple comparison test

In this study a higher levels of cells 8-OHG in the cells cultured for 24 and 48 h and then exposed to H_2O_2 alone for 2 h without any pretreatments with vitamin D; however when the cells

were pretreated with vitamin D for 24 or 48 h and then exposed to H₂O₂, they were completely protected from the induced oxidative stress as shown in Fig. 4a, b. The increased amount of 8-OHdG was found in both, cell pellet as well as the conditioned medium.

Polyubiquitin (polyUb) chain formation at DNA damage site:

Figure 5 showed an immunoblot with an antibody

against Ub. The DNA damage mark (polyUb) was significantly more in the whole cell lysate from H₂O₂ treated cells when compared to control cells. Cells that were pretreated with vitamin D for 24 h prior to H₂O₂ treatment lacked the polyUb mark. Tubulin was used as loading control where the polyUb signal could only be detected in the H₂O₂ treated cells.

DISCUSSION

The deficiency of vitamin D and increased oxidative stress has been well documented and is considered to be the leading cause of many neurodegenerative diseases¹⁵⁻¹⁷. The role of vitamin D in alleviating oxidative stress and conferring genomic stability and protection has been suggested, however, the exact molecular mechanisms that mediate this process are unclear. A study has shown that deficiency of vitamin D caused a greater degradation of protein and decreased synthesis of proteins in a muscle cell line, as well as all the antioxidant enzymes related to glutathione, such as glutathione peroxidase and glutathione reductase were brought to normal with the supplementation¹⁷ of vitamin D. It is believed that the immune-enhancing potential of vitamin D could lower immunosuppressive lipid peroxides, stabilize lysosomal membrane and protects nuclear structure¹⁰. Another mechanism through which vitamin D confers protection to the genomic stability is by raising the level of reduced glutathione¹⁸. Although these indirect ways of protecting the genomic integrity and stability of vitamin D are convincing, there is a need to look at the effect of oxidative

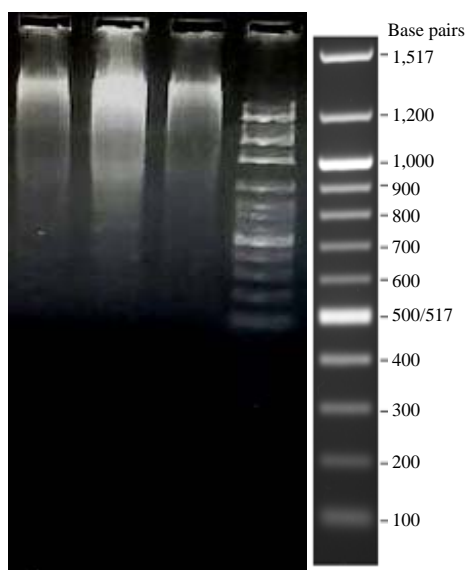


Fig. 3: Agarose gel for DNA damage
1.8% Agarose gel electrophoresis of genomic DNA obtained from control cultures, H₂O₂ treated cultures and the cultures pretreated with vitamin D for 24 h and then induced oxidative stress by H₂O₂

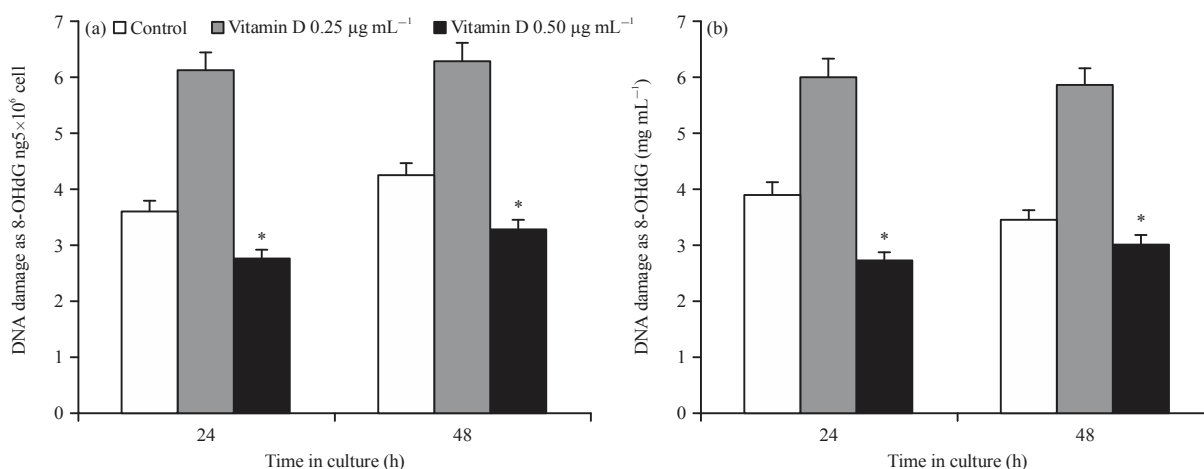


Fig. 4(a-b): Effect of vitamin D treatments on DNA damage in cortical neurons in culture, (a) Competitive enzyme linked immunosorbent assay was used to detect 8-OHdG in the cell pellet and (b) Secreted into the condition medium

The assay was done on cultures kept for 24 and 48 h with and without 0.25 µg mL⁻¹ of 1, 25 (OH)₂ D₃ followed by induced oxidative stress with H₂O₂ for 2 h, control cultures were grown without any treatments, *p<0.05 as significantly different from the H₂O₂ treated alone cultures

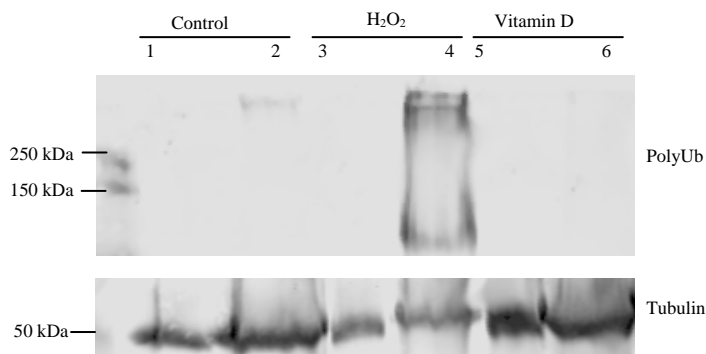


Fig. 5: Protective effect of vitamin D against DNA damage induced polyubiquitination

Western blot of lysates from control (1-2), H₂O₂ (3-4) and vitamin D (5-6) treated cells. Anti-PolyUb (top) and anti-Tubulin (bottom, as loading control). Lanes 2, 4 and 6 contain twice the protein amount loaded in 1, 3 and 5, respectively

stress directly on the DNA. This study was an attempt to study in detail the supplementation of vitamin D on neuronal cultures *in vitro* and the effect of induced oxidative stress on DNA and genomic stability.

The ROS are the intermediary metabolites of the cellular metabolism that produce peroxide and free radicals. Failure to detoxify these radicals can cause oxidative stress, the formation of abasic sites, purine or pyrimidine oxidation and DNA strand breaks¹¹. Oxidative stress can cause chromosomal aberration, abnormalities in chromosome structure that exhibit itself as acentric chromosomes fragments and asymmetrical re-arrangements resulting in the formation of dysenteric chromosomes¹⁹. Cells with lost or aberrant chromosomes have reduced survival, due to many cell cycle checkpoint arrest and programmed cell death and abnormal gene expression, which is to the benefit of the cell not to propagate any cells carrying gene mutations. In a study emulating hepatocellular carcinoma cells treated with diethyl-nitrosamine (DEN), a known carcinogen in the liver, an increased amount of DNA damage was reported. Treatment with vitamin D substantially reduced this DNA damage²⁰. In another study, synergistic application of both vanadium (an essential trace element) and vitamin D appears beneficial in preventing chromosomal aberrations and DNA strand breaks in rat liver when treated with the potent carcinogen²¹ DEN.

Recently a groundbreaking research proposed that the treatment with active metabolite of Vitamin D could result in complete restoration of 53BP1 to ensure genomic stability and proliferation in BRCA1 deficient breast cancer cell line²².

The results showed that PolyUb chain formation did not occur in cells treated with Vitamin D (24 h prior to H₂O₂ exposure) when compared to H₂O₂ treated cells. On the protein level, polyUb chain formation is a known marker of

DNA damage and is an essential step required for the DNA damage response. This study confirmed the polyubiquitination of unknown target proteins in response to H₂O₂. Vitamin D might prevent damage to DNA by upregulating or increasing the levels of monoubiquitinated histone H2A (ubH2A) upon exposure to H₂O₂ as seen by earlier work done by our group. Ongoing experiments are performed to further understand Vitamin D effect on the levels of ubH2A. It has been reported that the monoubiquitination of H2A is one of the first steps in the DNA damage response. This study suggests that by increasing the levels of ubH2A, local recruitment of DNA repair proteins, such as 53BP1 and BRCA1 is achieved on a lesser scale when compared to global DNA damage response. Therefore, the later polyubiquitination event is not needed due to the antioxidant neuroprotective properties of vitamin D.

CONCLUSION

The DNA damage response in the presence of vitamin D indicated that apart from its known function in bone formation and calcium homeostasis, it has a much wider and global role in cellular metabolism. Although further studies are warranted to elucidate the exact mechanism by which vitamin D confers the genomic stability and protection against oxidative stress. This study is of significance as it indicated the direct role of vitamin D in protecting against induced DNA damage by not needing the recruitment of DDR.

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