DOI: 10.7860/JCDR/2023/60147.17298

Original Article

Pharmacology Section

Antiproliferative and Proapoptotic Effects of Vitamin D3 in Human Neuroblastoma Cell Lines SH-SY5Y: An In-vitro Study

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ABSTRACT

Introduction: Neuroblastoma is the most common and earliest childhood tumour with complicated biological and clinical characteristics. The treatment includes chemotherapy, radiation therapy, surgical resection, stem cell therapy and many other modes, making the management difficult to tolerate and unacceptable. Thereby, increasing the need to develop novel therapies or repurpose already existing ones with anticancer potential. Many studies have shown that vitamin D3 has anticancer properties. Vitamin D3 receptors have been found in neuroblastoma cell lines, according to research. Anticancer property of vitamin D3 hasn't been studied much in neuroblastoma cell lines.

Aim: To evaluate the antiproliferative and proapoptotic effects of vitamin D3 on human neuroblastoma cell lines SH-SY5Y.

Materials and Methods: The present study was an in-vitro study in which human neuroblastoma cell lines SY5Y (a total of two cell lines) were obtained from National Centre for Cell Science (NCCS), Pune, Maharashtra, India, and control cells are cells from the cell lines that were left untreated. The antiproliferative effect of vitamin D3 in human neuroblastoma cell lines evaluated using the MTT (3-(4,5-dimethylthiazolyl-2)-2,5 diphenyltetrazolium bromide) assay. After 48 hours of incubation and treatment with six different (0.01, 0.1, 1, 10, 100, and 1000 ng/mL) concentrations of vitamin D3, the percentage of viable cells was determined using spectrophotometry and compared with control cells (untreated cells from cell lines). Different vitamin D3 (250 ng/mL, 500 ng/mL, 1000 ng/mL) doses were applied to cells and they were then incubated for 24 hours, Cell death and malformations were then observed using a phase contrast microscope, and Deoxyribonucleic Acid (DNA) fragmentation was investigated using gel chromatography. The obtained results were expressed as percentage of inhibition and tabulated in Microsoft Excel Sheet Version 16.16.27 and scatter plot graph was used to calculate IC_{50} (Half maximal inhibitory concentration).

Results: The vitamin D3 showed antiproliferative property in SH-SY5Y cells at an IC_{50} of 164 ng/mL when tested against human neuroblastoma cells using the MTT assay. Phase contrast microscope demonstrated that vitamin D3 treated cells showed condensation of nuclei, shrinkage of the cytoplasm, convolution of outline and cell peeling demonstrating apoptosis. DNA fragmentation also showed typical DNA ladder formation confirming apoptosis in vitamin D3 treated cells, which showed that the treated cells' DNA was more damaged than the control cells' DNA.

Conclusion: Vitamin D3 exhibited both proapoptotic and antiproliferative properties, as demonstrated by the MTT assay, Phase Contrast, and DNA fragmentation.

Keywords: Anticancer activity, Cholecalciferol, Half maximum inhibitory concentration

INTRODUCTION

Neuroblastoma is one of the most prevalent extracranial solid tumours in children with a prevalence rate of 10-15% of all the childhood tumours [1]. It is a complicated tumour with a variety of clinical and biological characteristics that arises from primordial neural crest cells [2]. It is hypothesised that morphological and bio-genetic characteristics may be beneficial in classifying children with neuroblastoma for proper care [3]. The tumour differs from other solid tumours due to its biological diversity, which ranges from spontaneous regression to aggressive metastatic illness [4]. The tumour is supposed to develop within the peripheral sympathetic nervous system's neuronal ganglia [5]. It is known that 60% of abdominal paraspinal ganglion tumours and 30% of neuroblastoma originate from the adrenal medulla that has led to the disease's clinical presentation being variable [6].

Understanding the intricate pathological diversity of the tumour by looking at its pathophysiology in connection to brain cells might assist uncover chemicals and, in turn, routes for targeted intervention [7]. The neural crest, also known as the fourth germ layer, is an embryological tissue that develops from neuroectoderm [8]. Through maturity, the first neural crest precursor develops multipotent differentiation potential and, as a result, acquires the ability to self-renew [9]. Many signaling pathways (like Bone Morphogenetic Protein (BMP), Wingless related integration site (Wnt), Notch, and others) are responsible for the cell differentiation [10]. Inhibiting these maturation pathways would likely make early multipotent neural crest precursors more likely to develop malignant transformation. In addition to this, numerous gene mutations have also been linked to the emergence of neuroblastoma [10]. Anaplastic Lymphoma Kinase (ALK), Protein Tyrosine Phosphate Non Receptor-11 (PTPN-11), Alpha-Thalassaemia/mental retardation, X-linked (ATRX), MYCN protooncogene (MYCN), and Neuroblastoma Rat Sarcoma (NRAS) gene alterations are the ones that are shown to be related with it most frequently [11].

Chemotherapy with drugs including vincristine, carboplatin, etoposide, cyclophosphamide, and cisplatin is the mainstay of treatment for neuroblastoma [12]. Further therapeutic options include surgical resection, radiotherapy, immunotherapy and other modes [12]. Based on the severity of the tumour, this multimodal therapy is divided into four phases: induction, local control, consolidation, and maintenance [13]. The total treatment time can take upto 18 months [14]. There is a need to develop innovative medicines with higher tolerability and acceptance because the existing therapy method is linked to adverse effects and low patient compliance [14].

Many malignant and non malignant cells have demonstrated significant physiological effects of vitamin D3 on proliferation and differentiation [15]. It is a strong seco-steroid with a significant impact on neuroprotection [15]. According to some studies, vitamin D3

may help to prevent neuronal damage brought on by various types of injury [15]. Several researches have shown that vitamin D3 has anticancer properties in prostate, colon, and breast cancer cell lines [16]. In the liver and kidney, vitamin D precursors are methodically converted into active hormone in two steps [17]. Vitamin D3 (cholecalciferol) and vitamin D2 are the two main physiologically inactive precursors of vitamin D (ergocalciferol) [17]. When they enter the liver, vitamin D precursors from the diet and sunlight exposure are both transformed to 25-hydroxyvitamin D [25(OH)D] (calcidiol) by the enzyme 25-hydroxylase [18]. The main form of vitamin D that circulates and is used to assess vitamin D levels is 25(OH)D. 1,25-dihydroxyvitamin D [1,25(OH)2D] requires further hydroxylation in the kidneys to become physiologically active (calcitriol) by the enzyme 1α-Hydroxylase [17,18]. The active hormone calcitrol interacts to the Vitamin D Receptor (VDR), which then affects the expression of several genes for skeletal and non skeletal homeostasis through a sequence of activation and co-activation events [19]. Several investigations have shown the existence of VDR in human neuroblastoma cell line [20]. Studies have also shown that VDR is necessary for vitamin D3's anticancer impact in cancer cells [21]. The amount of VDR present in the transfected cell lines correlated with the degree of vitamin D3-induced antiproliferative activity [21]. Vitamin D's antiproliferative effect results from its capacity to halt cell cycle progression [21]. P21 and other gene associated to the cell cycle have been shown to be directly impacted by vitamin D3 [22]. It has been demonstrated to suppress the Endothelial Growth Factor Receptor (EGFR) signaling cascade [23]. and inhibit the Hedgehog pathway [24]. By inhibiting the expression of antiapoptotic proteins including Bcl-2 and Bcl-xl and activating proapoptotic proteins BAX, BAK, and BAD, vitamin D3 has also been demonstrated to promote apoptosis in several cancer cells [25]. Vitamin D3 deficiency has been linked with many diseases including cancer, vitamin D3 has shown to be effective in treating many cancers including breast, prostate, squamous cell carcinoma and melanoma in-vitro [26]. The same principle has been applied in the present study to determine anticancer effects of vitamin D3 in neuroblastoma cell lines, which has not been explored much and in this study both antiproliferative and antiapoptotic effects of vitamin D3 has been explored.

The aim of the current study was to determine the antiproliferative effects of vitamin D3 against the human neuroblastoma cell line SH-SY5Y by examining its effects on cell viability percentage inhibition, cell migration inhibition, DNA damage inhibition, and apoptosis induction.

MATERIALS AND METHODS

The present study was an in-vitro study, which was carried out on the Human Neuroblastoma cell lines SH-SY5Y at Sri Ramachandra Medical College and Research Institute, Chennai, from December 2020-January 2021. The study has been carried out in SH-SY5Y cell lines which is a triple cloned cell line from a well-established SK-N-SH neuroblastoma cell lines (well established cell lines are usually cloned and cultured from already existing cell lines and not obtained directly from humans/animals) and have been obtained from NCCS, Pune , Maharashtra, India which produces and generates cultured and cloned cell lines. Since the cells haven't been harvested directly from humans/animals, Ethics approval was exempted for the present study as per the standard guidelines [27].

Chemicals and Reagents

Crystalline vitamin D3 was obtained from FERMENTA biotech, Thane, Maharashtra. DMSO (Dimethyl Sulfoxide) obtained from SRL (Sisco Research Laboratories) chemicals, Chennai. MTT, {3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)} from Sigma Aldrich, India.

The different media used are Foetal Bovine Serum (FBS), Ham's F12 medium and Minimum Essential Medium (MEM) which were obtained from Genetix Biotech, India.

Cell line

Human Neuroblastoma cell line, SH-SY5Y obtained from NCCS Pune, Maharashtra, India. The cell lines were cultured in an equal mixture of MEM and Ham's F12 medium which was supplemented with 10% FBS and antibiotics. For the control, the cells were left untreated.

Cytotoxicity assay (MTT) [28]: By using the MTT assay, the cytotoxicity of vitamin D3 was evaluated against the SHSY-5Y cell line [29]. The cells cultured with equal parts of MEM and F12 medium (that were treated with FBS, penicillin and streptomycin) at 37°C and allowed to grow to a convergence of 70-80%, post this they were plated in 96-well microplates (1×10^6 cells/wall), cultured for 24 hours at 37°C in 5% CO_2. After that, different Vitamin D3 concentrations (0.01, 0.1, 1, 10, 100, and 1000 ng/mL) were prepared from the stock (Vitamin D3 was prepared as 10 mg/mL stock by adding 100% ethanol), as serial dilution of 1:10 works well for initial experiment [30] and added to the medium and cells, and everything was incubated for 48 hours at 37°C in 5% CO_2. Following a Phosphate Buffer Saline (PBS, pH-7.3) wash, each well received 50 µL of MTT solution (5 mg/mL in PBS) and incubated for three hours. The plates were then let to sit for 30 minutes at 37° Celsius in the dark. The absorbance of the formazan crystals that gathered in well plates was measured after they were dissolved in 100 µL of DMSO spectrophotometrically at 570 nm. The following formula was used to compute the proportion of viable cells:

> Cell Viability (%)=(Absorbance of sample/Absorbance of control)×100 [31].

Phase contrast microscopy [32]: To study the morphological effects of vitamin D3 on the human neuroblastoma cell lines SH-SY5Y, phase contrast microscopy was used. Carefully sown in eight wells of six well plates, the neural cancer cell line (SHSY5Y) was allowed to adhere and proliferate for 24 hours. Vitamin D3 sample was supplied to the cells at dosage concentrations of 250 ng/mL, 500 ng/mL, and 1000 ng/mL (the reason for selecting 250 mg as dose was based on the IC_{50} value, based on the first dose the dose was doubled until response was obtained); each dose was given to the cells in two wells (duplicates) in maintenance media with 2% FBS. For 24 hours, the treated cell line was incubated. A phase contrast microscope was used to check the cells for cell death, deformities and cell shrinkage under 10X magnification, no special staining was done/used.

DNA fragmentation using agarose gel electrophoresis [33]: It was carried out to research how vitamin D3 affected the molecular behaviour of SH-SY5Y cell lines. Four T25 flasks were seeded with the cells, and they were given 24 hours to adhere and proliferate. Vitamin D3 was administered to the cells in maintenance media containing 2% FBS at dosage concentrations of 250 ng/mL, 500 ng/mL, and 1000 ng/mL. For 24 hours, the treated cell line was incubated. The cells were then removed for DNA isolation after being proteolysed. The manual Phenol-Chloroform DNA isolation process was used to isolate the DNA, and the pellet was then dissolved in nuclease-free water. A 0.8% agarose gel containing the DNA samples was loaded, and it was run at 50 volts. The gel was examined with a UV light source to look for DNA bands, which indicate DNA fragmentation.

STATISTICAL ANALYSIS

Obtained results were expressed as percentage of inhibition and Standard Deviation (SD) and it has been tabulated in Microsoft Excel Sheet Version 16.16.27. IC_{50} values are calculated using scattered plot graph.

RESULTS

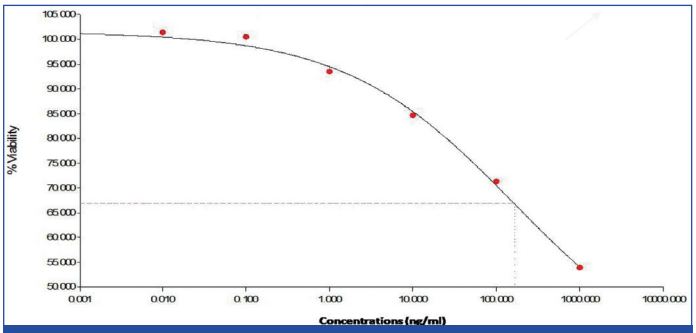
MTT Assay

Cell growth inhibition property: SH-SY5Y cells were screened against vitamin D3. A range of vitamin D3 concentrations (0.01, 0.1, 1, 10, 100, and 1000 ng/mL) were examined. Each measurement of vitamin D3 concentration was carried out four times, and the cumulative

difference between the data points was kept to under 20%, as shown in [Table/Fig-1]. A scatter plot in [Table/Fig-2] shows how at an IC_{50} of 164 ng/mL, which was obtained using the previously given equations.

Concentration of vitamin D3	% Viability	Standard deviation (Std.)
1000	53.9	3.89
100	71.3	6.03
10	84.7	4.51
1	93.5	5.79
0.1	100.5	2.14
0.01	101.4	5.39
Control	100.0	7.50

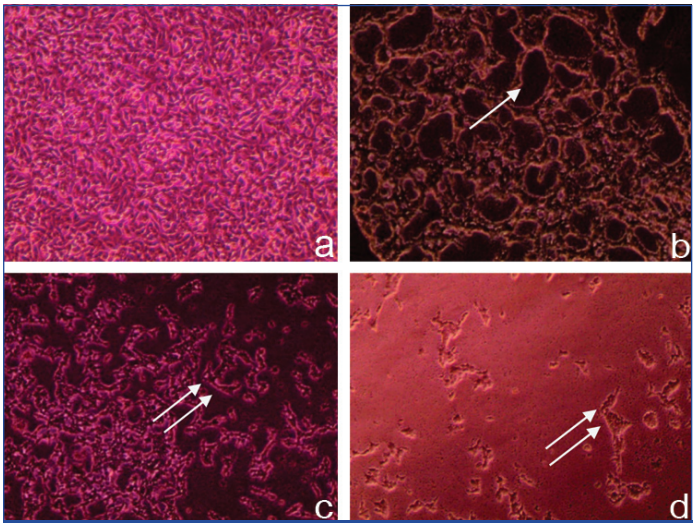
[Table/Fig-1]: Represents the percentage of viability of cells in treated and control cells. SH-SY5Y cells were cultured in the absence or presence of vitamin D3 for 24 hour as indicated in the materials and methods. Cell viability was determined based on the MTT assay. The columns represent %viability and standard deviation against the concentration of vitamin D3.



[Table/Fig-2]: Represents the curve of antiproliferative effect of vitamin D3 on SH-SY5Y cell lines as observed in MTT assay.

Phase Contrast

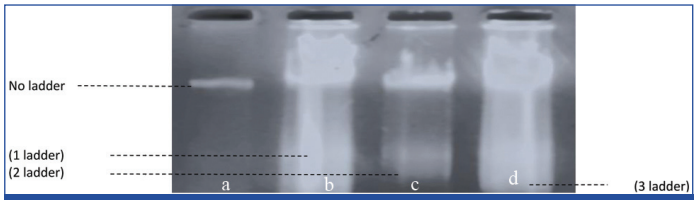
Vitamin D3 inhibited the proliferation of SH-SY5Y human neuroblastoma cells and caused them to undergo apoptosis, according to analysis utilising phase contrast microscopy. [Table/Fig-3a-d] demonstrates the action of different doses of vitamin D3 (250 ng/mL, 500 ng/mL and 1000 ng/mL) on treated cells as compared to the control (untreated cells). Blebbing of the membrane was seen with vitamin D3 and cell shrinkage was observed with increased dosage. With increasing vitamin D3 concentration, the number of cells with changed shape increased, reaching a maximum at 1000 ng/mL dose of vitamin D3.



[Table/Fig-3]: (a-d): Phase contrast Microscopy images of: (a) Control dose, (b) at a concentration of 250 ng/mL Vitamin D3 showed to cause blebbing of the membrane was seen with Vitamin D3 (single arrow), (c) 500 ng/ml cell shrinkage with chromatin condensation starting to appear (double arrow), and (d) 1000 ng/mL showing cell shrinkage (double arrow) and complete clearance of cells.

DNA Fragmentation

Vitamin D3-treated SH-SY5Y cells had shown morphological changes as well. DNA fragmentation was investigated by observing DNA ladder production. [Table/Fig-4a-d] illustrates how, after 48 hours of exposure, the DNA ladder became more noticeable with increasing dosages of vitamin D3 (250, 500, and 1000 ng/mL doses), which showed DNA fragmentation. However, the control group showed no signs of DNA fragmentation. (A DNA ladder/band is a piece of DNA that has undergone digestion and characterisation, yielding known base pair sizes. It is more evidence that vitamin D3 caused cell death via inducing apoptosis because DNA fragmentation is a characteristic of this process).



[Table/Fig-4]: DNA fragmentation on agarose gel electrophoresis: Lane (a) shows Control (untreated) lane no ladder formation seen, DNA ladder formation as seen on lane (b) Vitamin D3 dose of 250 ng/mL (1 ladder), Lane (c) Vitamin D3 dose of 500 ng/mL (2 ladders appreciated), and Lane (d) Vitamin D3 dose of 1000 ng/mL (3 ladders appreciated).

DISCUSSION

Neuroblastoma is a common tumour of paediatric age group with a complex management regimen and with a higher relapse rate in the post consolidation phase [34]. The presence of VDR in neuroblastoma cell lines, their quantities, and the active metabolite of vitamin D3 impacting the signalling via VDRs have all been connected to the mechanism of action of vitamin D3 in neuroblastoma cell lines programmed cell death (apoptosis) is a key tactic in the eradication of malignant cells [20]. In a review it has been noted that the prostate is a vitamin D3-target organ, which has supported the existence of VDR in prostate epithelial cells [35]. This resulted in the first in-vitro studies showing the antiproliferative effect of vitamin D3 on human CaP cells [35]. Additionally, vitamin D3 has been shown to have antiproliferative effects in some in-vitro experiments using prostate and breast cancer cell models [36].

The functional condition of the mitochondria is determined by the MTT colorimetric assay, which indicates cell viability. Living cells include an enzyme called mitochondrial dehydrogenase that converts yellow tetrazolium MTT salt to blue MTT formazan, which precipitates in healthy cells [37].The anticancer potential of vitamin D3 in numerous tumour types has been studied using MTT assay [38]. Vitamin D3 and its analogues has shown antiproliferative effects in an in-vitro investigation against the human breast cancer cell lines T47D and MCF-7, human epithelial squamous cell cancer cell line SCC-25, human leukaemia cell line HL-60, mouse derived leukaemia cell line WEHI-3, and mouse fibroblast cell line BALB/3T3 [38]. The idea that vitamin D3 binds to the VDR in epithelial cells and induces the differentiation of epidermal and leukaemic cells while also having antiproliferative properties against squamous cell cancer, breast cancer, and leukaemic cells in-vitro has been proven by this action [38].

Calcitrol (vitamin D3) showed to cause inhibition of 50% of cells (IC_{50}) in SCC-25 cell lines at a dose of 137 ng/mL, in MCF-7 breast cancer cell lines, IC_{50} of vitamin D3 was found to be at a dose of 43.6 ng/mL and in T47D cell lines it was found to be 67.7 ng/mL [38]. In the present study, the MTT assay indicated that vitamin D3 inhibits cell proliferation, with the IC_{50} of cell growth in the human neuroblastoma cell line being reached at a dosage of 164 ng/mL. In the present study, no positive control was used to compare the antiproliferarive effect of vitamin D3 using MTT assay as the main aim was to first establish the antiproliferative effect before the effect of vitamin D3 can be compared with a standard positive.

Apoptosis functions as a defence mechanism in healthy cells to get rid of damaged cells before they become cancerous [39]. Numerous chemotherapy medications work primarily by inducing apoptosis,

which kills malignant cell [40]. Apoptosis is a process that is characterised by morphological changes such as DNA fragmentation, chromatin condensation, membrane blebbing, cell shrinkage, and loss of mitochondrial membrane potential [41]. Extrinsic and intrinsic pathways have been identified as the two main types of apoptosis in living cells [41]. There are many methods to determine apoptosis in-vitro such as morphology-based methods, immunohistochemistry-based methods, biochemistry-based methods, immunology-based methods and array-based methods. Phase contrast microscopy is one type of morphology based method in which phase contrast microscope can be used to examine cells that have been cultured in flasks or plates. Dead cells start to float in the culture medium, as they separate from the substratum, to which they are attached. The phase contrast microscope makes it simple to identify these floating cells. A phase contrast microscope used with care can reveal blebs on apoptotic cells. Vacuoles that form in the cytoplasm indicate that the cells are still dispersed on the substratum which can be easily observed. Normally no special dyes are needed to stain the cells in phase constrast microscopy, in some specail cases fluoroscent dyes can be used to stain and observe the cells under phasse contrast microscopy [41]. Vitamin D3 has also shown to cause apoptosis in SH416–ER and PC9–ER lung cancer cell lines in a study done [42]. In this study, vitamin D3 has shown to cause apoptosis in SH-SY5Y cell lines by causing cell peeling, chromatin condensation, cell blebbing and other features of apoptosis.

Agarose gel electrophoresis is a type of biochemical method used for detection of apoptosis. The genomic DNA is divided into 180 base pairs or its multiples at specific locations (internucleosomal areas) during apoptosis, which causes it to appear like a ladder when electrophoresed. Because of this, this discovery is a distinctive, distinguishing property of apoptosis and is absent in necrosis (when cells are necrotic, they leave a smear pattern on the gel). As a result, it is one of the techniques used to distinguish between necrosis and apoptosis [41,43]. Vitamin D3 has shown to cause apoptosis in human breast cancer cell lines MCF-7, MDA-MB-435 cell lines, human prostate cancer cell lines LnCaP and Human Osteosarcoma cell lines U2OS, and has shown DNA ladder formation in them [44]. In this study, vitamin D3 has shown to cause DNA laddering in human neuroblastoma cell lines-SH-SY5Y under agarose gel electrophoresis.

One study has observed that vitamin D3 has shown to have proliferative effects on normal germline related stemcells (Very Small Embryonic Like Stem cells- VSELS) but at the same time it is inhibitory to tumour cells, which is correlated to the pleotropic effects of vitamin D3 in normal and cancerous cells, thereby, making supplementation with vitamin D3 important for both normal and pathological conditions [45].

Despite extensive research examining vitamin D3's anticancer effectiveness in many tumour forms, very few studies have examined its impact on neuroblastoma. In the present study, from the results has been clearly demonstarted that vitamin D3 has proapoptotic effects as evidenced by cell shrinkage, nuclear condensation, membrane degradation, and cell peeling, chromatin clevage and cell death under the phase contrast microscopy and this was confirmed by typical laddering of DNA fragmnents formed using the DNA fragmentation on agarose gel electrophoresis, which is considered to be the biochemical hallmark of apoptosis.

With these findings, it can be definitively stated that vitamin D3 inhibits cell proliferation while promoting apoptosis in the human neuroblastoma SH-SY5Y cell line. But, more assays need to be done to strongly establish the role of vitamin D3 in neuroblastoma. With this study it can be implied that the anticancer property of vitamin D3 can be used in the future to establish it, as an adjunctive therapy with the standard chemotherapy of neuroblastoma, provided it shows similar positive effects in future in-vivo and clinical trials.

Limitation(s)

The present study's limitation was the lack of a positive control, which would have allowed vitamin D3's antiproliferative activity to be amplified.

CONCLUSION(S)

Within the limits of the present study, it has been concluded that vitamin D3 has demonstrated antiproliferative and proapoptotic activity with the help of the MTT assay, phase contrast microscopy, and DNA fragmentation, in human neuroblastoma cell line SH-SY5Y. However, to strengthen the proof of vitamin D3's anticancer effect in human neuroblastoma, more tests and in-depth research must be conducted both in-vitro and in-vivo.

Acknowledgement

The authors are thankful to the Department of Pharmacology, SRMC&RI, Porur, Chennai, Tamil Nadu, India.

REFERENCES

[1] Ponzoni M, Bachetti T, Corrias MV, Brignole C, Pastorino F, Calarco E, et al. Recent advances in the developmental origin of neuroblastoma: An overview. J Experi Clin Cancer Res. 2022;41(1):01-28.
[2] Pizzo PA, Poplack DG. Principles and practice of pediatric oncology. Lippincott Williams & Wilkins; 2015 .
[3] Riley RD, Heney D, Jones DR, Sutton AJ, Lambert PC, Abrams KR, et al. A systematic review of molecular and biological tumor markers in neuroblastoma. Clinical Cancer Research. 2004;10(1):04-12.
[4] Park JR, Bagatell R, London WB, Maris JM, Cohn SL, Mattay KM, et al. COG Neuroblastoma Committee. Children's Oncology Group's 2013 blueprint for research: Neuroblastoma. Pediatric Blood & Cancer. 2013;60(6):985-93.
[5] Betters E, Liu Y, Kjaeldgaard A, Sundström E, García-Castro MI. Analysis of early human neural crest development. Developmental Biology. 2010;344(2):578-92.
[6] Fredlund E, Ringnér M, Maris JM, Påhlman S. High Myc pathway activity and low stage of neuronal differentiation associate with poor outcome in neuroblastoma. Proceedings of the National Academy of Sciences. 2008;105(37):14094-99.
[7] Wang Y, Luo H, Cao J, Ma C. Bioinformatic identification of neuroblastoma microenvironment-associated biomarkers with prognostic value. J Oncol. 2020;2020.
[8] Hall BK. Germ layers, the neural crest and emergent organization in development and evolution. Genesis. 2018;56(6-7):e23103.
[9] Prasad MS, Sauka-Spengler T, LaBonne C. Induction of the neural crest state: Control of stem cell attributes by gene regulatory, post-transcriptional and epigenetic interactions. Developmental Biology. 2012;366(1):10-21.
[10] Zhang Y, Weinberg RA. Epithelial-to-mesenchymal transition in cancer: Complexity and opportunities. Frontiers Med. 2018;12(4):361-73.
[11] Depuydt P, Boeva V, Hocking TD, Cannoodt R, Ambros IM, Ambros PF, et al. Genomic amplifications and distal 6q loss: Novel markers for poor survival in high-risk neuroblastoma patients. JNCI: Journal of the National Cancer Institute. 2018;110(10):1084-93.
[12] Forouzani-Moghaddam MJ, Nabian P, Gholami A, Dehghanbaghi N, Azizipanah M, Jokar K, et al. A review of neuroblastoma: Prevalence, diagnosis, related genetic factors, and treatment. Iranian Journal of Pediatric Hematology and Oncology. 2018;8(4):237-46.
[13] Bini F, Frati A, Garcia-Gil M, Battistini C, Granado M, Martinesi M, et al. New signalling pathway involved in the anti-proliferative action of vitamin D3 and its analogues in human neuroblastoma cells. A role for ceramide kinase. Neuropharmacology. 2012;63(4):524-37.
[14] Pinto NR, Applebaum MA, Volchenboum SL, Matthay KK, London WB, Ambros PF, et al. Advances in risk classification and treatment strategies for neuroblastoma. J Clin Oncol. 2015;33(27):3008.
[15] Tetich M, Kutner A, Leskiewicz M, Budziszewska B, Lasoń W. Neuroprotective effects of (24R)-1, 24-dihydroxycholecalciferol in human neuroblastoma SH-SY5Y cell line. J Steroid Biochem Mol Biol. 2004;89:365-70.
[16] Markowska A, Antoszczak M, Kojs Z, Bednarek W, Markowska J, Huczyński A. Role of vitamin D3 in selected malignant neoplasms. Nutrition. 2020;79:110964.
[17] Jeon SM, Shin E. Exploring vitamin D metabolism and function in cancer. Experimental Mol Med. 2018;50(4):01-04.
[18] Zhang R, Naughton DP. Vitamin D in health and disease: Current perspectives. Nutrition J. 2010;9(1):01-03.
[19] Trump DL, Aragon-Ching JB. Vitamin D in prostate cancer. Asian J Andrology. 2018;20(3):244.
[20] Harms LR, Burne TH, Eyles DW, McGrath JJ. Vitamin D and the brain. Best Pract Res Clin Endocrinol Metab. 2011;25(4):657-69.
[21] Chiang KC, C Chen T. The anti-cancer actions of vitamin D. Anti-cancer agents in medicinal chemistry (Formerly Current Medicinal Chemistry-Anti-Cancer Agents). 2013;13(1):126-39.
[22] Bhoora S, Punchoo R. Policing cancer: Vitamin D arrests the cell cycle. Int J Mol Sci. 2020 ;21(23):9296.
[23] Ferrer-Mayorga G, Larriba MJ, Crespo P, Muñoz A. Mechanisms of action of vitamin D in colon cancer. J Steroid Biochem Mol Biol. 2019;185:01-06.

[24] Uhmann A, Niemann H, Lammering B, Henkel C, Heß I, Nitzki F, et al. Antitumoral effects of calcitriol in basal cell carcinomas involve inhibition of hedgehog signaling and induction of vitamin D receptor signaling and differentiation. Mol Cancer Ther. 2011;10(11):2179-88.
[25] Adelani IB, Rotimi OA, Maduagwu EN, Rotimi SO. Vitamin D: Possible therapeutic roles in hepatocellular carcinoma. Frontiers in Oncology. 2021;11:1391.
[26] Feles S, Overath C, Reichardt S, Diegeler S, Schmitz C, Kronenberg J, et al. Streamlining culture conditions for the neuroblastoma cell Line SH-SY5Y: A prerequisite for functional studies. Methods and Protocols. 2022;5(4):58.
[27] Geraghty RJ, Capes-Davis A, Davis JM, Downward J, Freshney RI, Knezevic I, et al. Guidelines for the use of cell lines in biomedical research. Br J Can. 2014;111(6):1021-46.
[28] Dantu AS, Shankarguru P, Ramya DD, Vedha HB. Evaluation of in vitro anticancer activity of hydroalcoholic extract of *Tabernaemontana divaricata*. Asian J Pharm Clin Res. 2012;5(3):59-61.
[29] Carmichael J, DeGraff WG, Gazdar AF, Minna JD, Mitchell JB. Evaluation of a tetrazolium-based semiautomated colorimetric assay: Assessment of chemosensitivity testing. Cancer Res. 1987;47(4):936-42.
[30] https://www.texaschildrens.org/sites/default/files/pictures/departments/Cancer/horton-mtt-cell-assay-protocol1.pdf.
[31] Patel S, Gheewala N, Suthar A, Shah A. In-vitro cytotoxicity activity of Solanum nigrum extract against Hela cell line and Vero cell line. Int J Pharm PharmSci. 2009;1(1):38-46.
[32] Li W, Song K, Wang S, Zhang C, Zhuang M, Wang Y, et al. Anti-tumor potential of astragalus polysaccharides on breast cancer cell line mediated by macrophage activation. Materials Science and Engineering. 2019;98:685-95.
[33] Jeon SM, Shin E. Exploring vitamin D metabolism and function in cancer. Experimental & Mol Med. 2018;50(4):01-04.
[34] Pizzo RA. Solid tumors of childhood. Cancer: Principles and practice of oncology. 1993.
[35] Feldman D, Zhao XY, Krishnan AV. Editorial/mini-review: Vitamin D and prostate cancer. Endocrinology. 2000;141(1):05-09.
[36] Yoshioka M. Downregulation of MYCN oncogene by retinoic acid in neuroblastoma (Doctoral dissertation, University of Hawai'i at Manoa).
[37] Vajrabhaya LO,Korsuwannawong S. Cytotoxicity evaluation of a Thai herb using tetrazolium (MTT) and sulforhodamine B (SRB) assays. Journal of Analytical Science and Technology. 2018;9(1):01-06.
[38] Wietrzyk J, Chodynski M, Fitak H, Wojdat E, Kutner A, Opolski A, et al. Antitumor properties of diastereomeric and geometric analogs of vitamin D3. Anti-cancer Drugs. 2007;18(4):447-57.
[39] Kaufmann SH, Hengartner MO. Programmed cell death: Alive and well in the new millennium. Trends in Cell Biology. 2001;11(12):526-34.
[40] Zeinoddini S, Nabiuni M, Jalali H. The synergistic cytotoxic effects of doxorubicin and Viola odorata extract on human breast cancer cell line T47-D. J Can Res Therapeutics. 2019;15(5):1073.
[41] Ulukaya E, Acilan C, Ari F, Ikitimur E, Yilmaz Y. A glance at the methods for detection of apoptosis qualitatively and quantitatively. Turkish Journal of Biochemistry/Turk BiyokimyaDergisi. 2011;36(3).
[42] Shaurova T, Dy GK, Battaglia S, Hutson A, Zhang L, Zhang Y, et al. Vitamin D3 metabolites demonstrate prognostic value in EGFR-mutant lung adenocarcinoma and can be deployed to oppose acquired therapeutic resistance. Cancers. 2020;12(3):675.
[43] Majtnerová P, Roušar T. An overview of apoptosis assays detecting DNA fragmentation. Molecular biology reports. 2018;45(5):1469-78.
[44] Fife RS, Sledge Jr GW, Proctor C. Effects of vitamin D3 on proliferation of cancer cells in vitro. Cancer letters. 1997;120(1):65-69.
[45] Abdelbaset-Ismail A, Pedziwiatr D, Suszyńska E, Sluczanowska-Glabowska S, Schneider G, Kakar SS, et al. Vitamin D3 stimulates embryonic stem cells but inhibits migration and growth of ovarian cancer and teratocarcinoma cell lines. Journal of Ovarian Research. 2016;9(1):01-02.

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PLAGIARISM CHECKING METHODS: [Jain H et al.]
- Plagiarism X-checker: Sep 16, 2022
- Manual Googling: Sep 23, 2022
- iThenticate Software: Dec 01, 2022 (11%)

ETYMOLOGY: Author Origin

AUTHOR DECLARATION:
- Financial or Other Competing Interests: None
- Was Ethics Committee Approval obtained for this study? No
- Was informed consent obtained from the subjects involved in the study? No
- For any images presented appropriate consent has been obtained from the subjects. NA

Date of Submission: **Sep 09, 2022**
Date of Peer Review: **Oct 17, 2022**
Date of Acceptance: **Dec 03, 2022**
Date of Publishing: **Jan 01, 2023**