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Original article

Role of vitamin D₃ on apoptosis and inflammatory-associated gene in colorectal cancer: An *in vitro* approach

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ABSTRACT

The association between vitamin D and cancer has been studied with different focuses. As far as the beneficial effects concern, studies have shown the effects of vitamin D in inflammatory response and oxidative stress in relation with cancer regression. Indirect anti-cancer effects of vitamin D also due to its anti-inflammatory properties. Nuclear factor kappa B (NF- κ B) is considered as a major mediator of inflammation and key transcription factor linking inflammation of cancer development. On the other hand, caspase protein also has an important role in apoptosis. Further, apoptosis and the BCL-2 family now inextricably linked to human cancer. Since the vitamin D₃ has proven to exert numerous tumor suppressive effects, the relationship of vitamin D₃ on the regulation of apoptosis and NF- κ B is yet to be studied in detail. Hence, the present study aims at evaluating the efficacy of vitamin D₃ on NF- κ B and Caspase 3 in the human colorectal carcinoma cell line (HCT116). Treatment of HCT cell line with different concentration of vitamin D₃ shown a dose-dependent effect on the expression of Caspase 3 not on NF- κ B. This finding suggests that vitamin D does not have any effect on NF- κ B, but have a positive effect on Caspase3 on HCT116, which might play a vital role in preventing the colon cancer.

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1. Introduction

Vitamin D was discovered in 1919 during rickets experiments by Edward Mellanby (Mehta and Mehta, 2002; Mellanby, 1919). There are two main forms of vitamin D exist in nature: Photochemically synthesized vitamin D₂ (ergocalciferol) in plants, and vitamin D₃ (cholecalciferol) synthesized in the skin of animals and humans (Vuolo et al., 2012; Norman, 1998). According to recent research, vitamin D may play an important role in preventing and treating many diseases like type 1 and type 2 diabetes

(Hyppönen et al., 2001; Pittas et al., 2006), hypertension (Krause et al., 1998), glucose intolerance (Chiu et al., 2004), multiple sclerosis (Munger et al., 2006) and other medical conditions like rheumatoid arthritis (Merlino et al., 2004; Schleithoff et al., 2006).

Cancer is a disease induced by genetic changes that result in uncontrolled development of cells and the formation of tumors. When considering the relationship between cancer and beneficial effect of vitamin D₃, different opinions exist in recent years. Many, although not all, studies that associate vitamin D levels with cancer risk measure serum levels of 25(OH)D to determine if the subjects are deficient. For example, a greater proportion of melanoma patients had deficient or insufficient levels of 25(OH)D than healthy controls (Cattaruzza et al., 2019). Similarly, vitamin D levels were lower in patients with thyroid cancer compared to matched controls (Heidari et al., 2017). Women with breast cancer that were deficient in 25(OH)D had larger tumors, more advanced stage and reduced survival than those that were not deficient (Ismail et al., 2018).

Not all studies have shown beneficial effects of vitamin D and/or D₃ supplementation in cancer treatment. Experimental evidence

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of vitamin D has also shown that it may reduce the risk of cancer by regulating anti-proliferative effect, apoptosis, stimulation of differentiation, anti-inflammatory effect, inhibition of invasion and metastasis, and the inhibition of angiogenesis (Liu et al., 2018). Emerging epidemiological data suggest that vitamin D may have a protective effect against colon cancer, but there is no strong evidence for prostate, breast and other cancers (Davis, 2008; Davis et al., 2007). Previous study shown not only the beneficial effect of vitamin D on cancer treatment but has also shown no effect or even increased cancer risk as well. The reasons for such variability is because of multiple attribute in study design and approaches. Therefore, adequate research is needed to determine whether the inadequacy of vitamin D can lead to increased risk of cancer or not (Davis et al., 2007). Hence, the present study aims at investigating the efficacy of vitamin D₃ as a preventive and/or therapeutic agent for colon cancer through targeting specific gene of interest.

2. Materials and methodology

2.1. Cell culture

Human colorectal carcinoma cell line, HCT116, was obtained from National Centre for Cell Science (NCCS) India which was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% antibiotics (Penicillin and Streptomycin) in an incubator with the presence of 5% CO₂ at 37 °C. The media were changed at regular intervals and when the cells reached the 80% confluence, the cells were seeded onto 6-well plate at a density of 1X10⁵ for further treatment and analysis.

2.2. MTT assay

MTT Assay was performed to find out the viability and IC₅₀ value at different concentrations of vitamin D₃ (Merck) such as 2.5 μM, 5 μM, 7.5 μM, 10 μM, 15 μM, 20 μM, 40 μM. Briefly, cells were seeded on 96-well plate and treated with indicated concentrations of vitamin D₃ at 80% confluence level for 24 h. At the end of the experiment, MTT was added and read at 590 nm for the absorbance.

2.3. RT-PCR and gene expression

To depict the beneficial effect of vitamin D₃ as prevention and/or therapeutic agent for colon cancer, the HCT119 cells were cultured on 6-well plate and treated with different concentration of vitamin D₃ viz 5 μM, 15 μM and 20 μM (dosage was fixed based on the MTT assay) for 24 h. At the end of the experiment, cells were triplicated and total RNA was isolated by the Trizol method as previously described (Rio et al., 2010). Approximately, 2 μg of RNA was used for cDNA synthesis using a PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa Bio Inc., Japan) by reverse transcriptase PCR (Cyberlab, USA). For the expression of *Caspase 3* in the treated cell line, the primers 5'-AGTAGATGGTTTGAGCCTGAGC-3' (Forward) and 5'-TTTCTTACTTGGCGATGGCG-3' (Reverse) was used. Similarly, for *NF-κB*, 5'-CCCACGAGCTGTAGAAAGG-3' (Forward) and 5'-CTGGATGCGCTGACTGATAG-3' (Reverse) primers was used. The house keeping gene, β actin, was used as internal control and the primers used for actin was 5'-AAGAGAGGCATCTCACCT-3' (Forward) and 5'-TACATGGCTGGGTGTGAA-3' (Reverse). The intensity of the band was calculated using image pro software and the results obtained from three different experiments were used for the expression analysis.

3. Results

In this present investigation, we evaluated the toxicity of vitamin D₃ through percentage of cell viability upon treatment with different concentrations as shown in Fig. 1. The result shown that the viability of cells treated with vitamin D₃ decreases with increasing concentrations. The higher the concentration (40 μM) shows the maximum of 20% cell viability approximately. Hence, vitamin D₃ has a significant effect on cell survival in a dose-dependent manner (Fig. 1). Further, the IC₅₀ value of vitamin D₃ observed at a concentration of 15 μM in HCT116 cell line.

3.1. Expression of Caspase 3 in HCT116

To know the effect of vitamin D₃ on expression of *Caspase 3*, HCT116 cells were treated vitamin D₃ as shown in Fig. 2. In this, the cells were treated with three different concentrations of vitamin D₃ as mentioned along with DMSO as a vehicle control. The cells treated with vitamin D₃ has significant upregulation of *Caspase 3* with respect to the vitamin D₃ concentration. On the other hand, a dose-dependent increase in the expression of *Caspase 3* observed in the present investigation (Fig. 2A and B).

3.2. Expression of NF-κB in HCT116 treated with vitamin D₃

We further intended to investigate if vitamin D₃ has a beneficial effect on the inflammatory response in colon cancer. Hence, the HCT116 cells treated with the different concentrations of vitamin D₃ as shown in Fig. 3. The expression of *NF-κB* was observed among the treated cells. However, the cells treated with vitamin D₃ shows no significant changes in the expression of *NF-κB* when compared with each other (Fig. 3A and B).

4. Discussion

In this present study, a dose-dependent effect of vitamin D₃ on the expression of *Caspase 3* and *NF-κB* was performed to make out beneficial role of vitamin D₃ in cancer prevention on HCT116. MTT assay has depicted the viability of cells and also the IC₅₀ value of vitamin D₃. Cells treated with different concentrations of vitamin D₃ such as 2.5 μM, 5 μM, 7.5 μM, 10 μM, 15 μM, 20 μM and 40 μM shows the significant changes in cell viability with increased concentrations of vitamin D₃, which suggest that vitamin D₃ might play a role in cell homeostasis. Further, the IC₅₀ value of vitamin D₃ was found to be at a concentration of 15 μM. Few studies shown similar type of experiments in which the cells treated with vitamin D have lesser significant results. Shruithi et al., (2017) finds the cellular uptake of vitamin D by the HCT116 treated with different

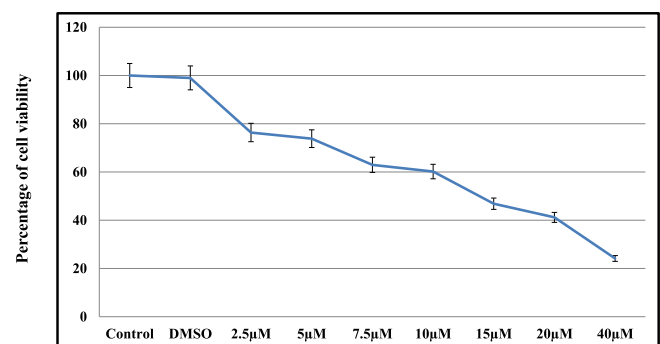


Fig. 1. MTT assay for the percentage of cell viability in HCT119 cell lines which treated with different indicated concentrations of vitamin D₃. values for the percentage indicates mean ± SD of three different experiments.

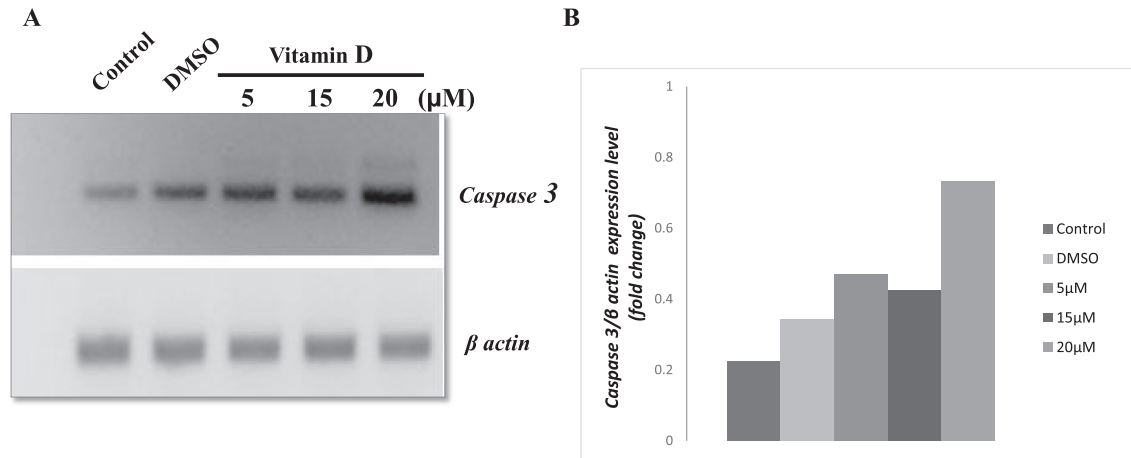


Fig. 2. (A) RT-PCR for *Caspase 3* gene expression in HCT119 treated with different concentrations of vitamin D₃. (B) Represents the densitometry analysis for the level of gene expression among vitamin D₃ treated and control groups. The values shown are obtained from there different experiments. β actin served as internal control.

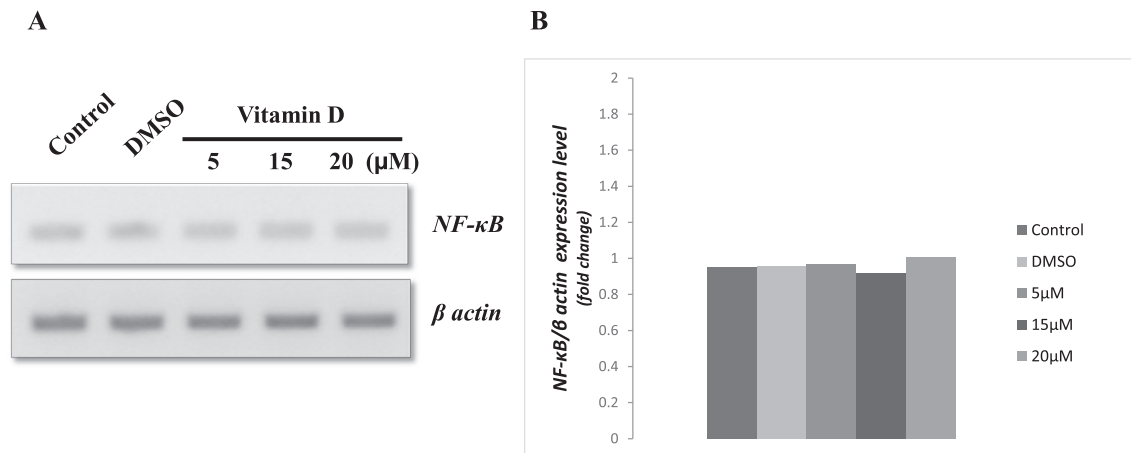


Fig. 3. (A) RT-PCR for *NF-κB* gene expression in HCT119 treated with different concentrations of vitamin D₃. (B) Represents the densitometry analysis for the level of gene expression among vitamin D₃ treated and control groups. The values shown are obtained from there different experiments. β actin served as internal control.

concentration of vitamin D₃. Similarly, [Diesing et al. \(2006\)](#) used supraphysiological concentration and physiological concentration to find cellular uptake of vitamin D and confirmed the uptake of vitamin D by cancer cells. In respect with this result and evident, HCT116 cells were treated with the three different concentration to understand the effect on the gene expression such as *Caspase 3* and *NF-κB*. The results suggest that the supplement of vitamin D₃ increase apoptotic regulatory protein *Caspase 3* expression, but this result is not enough to prove the effect on *NF-κB*. Previous studies have demonstrated that the *NF-κB* has a very important role in the CRC as CRC patients will develop inflammation ([Barbáchano et al., 2018](#)). It has been shown that vitamin D₃ reduce the expression of *NF-κB* suggesting that vitamin D₃ has an anti-inflammatory property which will gradually reduce the CRC from further developing into inflammation. However, our results are not consistent with this study and do not provide a valid conclusion on this part. Because, *NF-κB* is inhibited by vitamin D₃ through several pathways and it may depend on the types of cancer as well.

The expression of *Caspase 3* shows there is an increased expression of the genes as of concentration of vitamin D₃ increases. This result further suggests that vitamin D₃ regulates on the expression of *Caspase 3*, an important mediator of apoptosis. Since apoptosis is a major target for control of cancer growth, the increased index of *Caspase 3* in vitamin D₃-HCT116 might have a beneficial role on

colon carcinoma as a homeostatic regulator. Previous report suggests that the treatment with the active component of vitamin D₃ will cause apoptosis through the increase in several pro-apoptotic proteins, decrease in anti-apoptotic proteins and associated proteins ([Barbáchano et al., 2018](#)). In support with the previous data, our result suggests that vitamin D₃ upregulates *Caspase 3* which in turn regulate apoptosis in colon cancer.

The present study also investigated the effect of vitamin D₃ on the inflammatory and apoptotic factors on the human colon carcinoma cell HCT116. Vitamin D₃ has direct or indirect effect as anti-inflammatory ([Lin and Li, 2016](#)) and other cellular processes like anti-proliferative, stimulation of differentiation, inhibition of metastasis and invasion and inhibition of angiogenesis ([Liu et al., 2018](#)). Previous studies have focused on the anti-inflammatory and inhibition of apoptosis property. As both the factors are important for reducing the CRC, we focused on *NF-κB* and *Caspase 3*. In CRC patients, inflammation is a major factor which in severe cases does not have treatment. The active component of vitamin D known as the calcitriol exerts anti-inflammatory properties and inhibits IL-1 β , IL-6, IL-8, TNF- α and possibly other cytokines. Further, indirect anti-cancer effects of vitamin D can also be due to its anti-inflammatory properties. Men with prostate cancer had reduced 25(OH)D and increased inflammatory mediator levels compared to controls ([Xie et al., 2017](#)). The results of our study indicate that *NF-κB* has no significant changes in their expression

in vitamin D₃ treated HCT119 cell line. This observation may be because of sufficient supplementation of vitamin D₃ concentration.

On the whole, we conclude that vitamin D₃ does not affect the level of *NF-κB* in a particular concentration. At the same time, it was found that *Caspase 3* is upregulated in the vitamin D₃ treated cells in a dose-dependent manner. Thus, from this present study, we conclude that vitamin D₃ could promote apoptosis in the HCT116. However, there is need of more supporting experimental evidence for the use of vitamin D₃ as a potential therapeutic agent for the treatment of colorectal cancer.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

- Barbáchano, A., Larriba, M.J., Ferrer-Mayorga, G., González-Sancho, J.M., Muñoz, A., 2018. Vitamin D and colon cancer. In *Vitamin D*, Academic Press-Elsevier. 2(99), 837–862.
- Cattaruzza, M.S., Pisani, D., Fidanza, L., Gandini, S., Marmo, G., Narcisi, A., Bartolazzi, A., Carlesimo, M., 2019. 25-Hydroxyvitamin D serum levels and melanoma risk: a case-control study and evidence synthesis of clinical epidemiological studies. *Eur. J. Can. Prev.* 28 (3), 203–211.
- Chiu, K.C., Chu, A., Go, V.L., Saad, M.F., 2004. Hypovitaminosis D is associated with insulin resistance and β cell dysfunction. *Am. J. Clin. Nut.* 79 (5), 820–825.
- Davis, C.D., 2008. Vitamin D and cancer: current dilemmas and future research needs. *Am. J. Clin. Nut.* 88 (2), 565S–569S.
- Davis, C.D., Hartmuller, V., Freedman, D.M., Hartge, P., Picciano, M.F., Swanson, C.A., Milner, J.A., 2007. Vitamin D and cancer: Current dilemmas and future needs. *Nutr. Rev.* 65 (8Pt2), S71–S74.
- Diesing, D., Cordes, T., Fischer, D., Diedrich, K., Friedrich, M., 2006. Vitamin D-metabolism in the human breast cancer cell line MCF-7. *Anticancer Res.* 26, 2755–2760.
- Heidari, A., Nikbakht, M., Mashhadi, M.A., Jahantigh, M., Mansournia, N., Sheikhi, V., Mansournia, M.A., 2017. Vitamin D deficiency associated with differentiated thyroid carcinoma: a case-control study. *As. Pac. J. Can. Preven.* 18 (12), 3419–3422.
- Hyppönen, E., Läärä, E., Reunanen, A., Järvelin, M.R., Virtanen, S.M., 2001. Intake of vitamin D and risk of type 1 diabetes: a birth-cohort study. *The Lancet* 358 (9292), 1500–1503.
- Ismail, A., El-Adawy, R., Mohamed, G., Hussenin, M., Ramadan, S.S., 2018. Prognostic significance of serum vitamin D levels in Egyptian females with breast cancer. *As. Pac. J. Can. Preven.* 19 (2), 571–576.
- Krause, R., Bühring, M., Hopfenmüller, W., Holick, M.F., Sharma, A.M., 1998. Ultraviolet B and blood pressure. *Lancet (British edition)* 352 (9129), 709–710.
- Lin, Z., Li, W., 2016. The role of vitamin D and its analogs in inflammatory disease. *Curr. Topics Med. Chem.* 16 (11), 1242–1261.
- Liu, W., Zhang, L., Xu, H.-J., Li, Y., Hu, C.M., Yang, J.Y., Sun, M.Y., 2018. The anti-inflammatory effects of vitamin D in tumorigenesis. *Int. J. Mol. Sci.* 19 (9), 2736.
- Mehta, R.G., Mehta, R.R., 2002. Vitamin D and cancer. *J. Nut. Biochem.* 13 (5), 252–264.
- Mellanby, E., 1919. An experimental investigation of rickets. *Lancet* 1, 407–412.
- Merlino, L.A., Curtis, J., Mikuls, T.R., Cerhan, J.R., Criswell, L.A., Saag, K.G., 2004. Vitamin D intake is inversely associated with rheumatoid arthritis: results from the Iowa Women's Health Study. *Arthritis Rheumatism* 50 (1), 72–77.
- Munger, K.L., Levin, L.L., Hollis, B.W., Howard, N.S., Ascherio, A., 2006. Serum 25-hydroxyvitamin D levels and risk of multiple sclerosis. *JAMA* 296 (23), 2832–2838.
- Norman, A.W., 1998. Sunlight, season, skin pigmentation, vitamin D, and 25-hydroxyvitamin D: integral components of the vitamin D endocrine system. *Am. J. Clin. Nut.* 67 (6), 1108–1110.
- Pittas, A.G., Dawson-Hughes, B., Li, T., Van Dam, R.M., Willett, W.C., Manson, J.E., Hu, F.B., 2006. Vitamin D and calcium intake in relation to type 2 diabetes in women. *Diab. Care.* 29 (3), 650–656.
- Rio, D.C., Ares, M., Hannon, G.J., Nilsen, T.W., 2010. Purification of RNA using TRIzol (TRI reagent). *Cold Spring Harbour Protocol* 2010 (6), 2010.
- Schleithoff, S.S., Zittermann, A., Tenderich, G., Berthold, H.K., Stehle, P., Koerfer, R., 2006. Vitamin D supplementation improves cytokine profiles in patients with congestive heart failure: a double-blind, randomized, placebo-controlled trial. *Am. J. Clin. Nut.* 83 (4), 754–759.
- Shruthi, N., Prashanthkumar, M.V., Venugopalreddy, B., Suma, M.N., Subba Rao, V. M., 2017. Analysis of the cytotoxic effects of vitamin D₃ on colorectal, breast and cervical carcinoma cell lines. *Biochem. Anal. Biochem.* 6 (2).
- Vuolo, L., Di Somma, C., Faggiano, A., Colao, A., 2012. Vitamin D and cancer. *Front. Endocrin.* 3.
- Xie, D.D., Chen, Y.H., Xu, S., Zhang, C., Wang, D.M., Wang, H., Chen, L., Zhang, Z.H., Xia, M.Z., Xu, D.X., Yu, D.X., 2017. Low vitamin D status is associated with inflammation in patients with prostate cancer. *Oncotarget* 8 (13), 22076–22085.