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IN VITRO ANALYSIS OF MELANOMA CELL PROLIFERATION SUBJECTED TO HIGH DOSES OF VITAMIN D

ANÁLISE IN VITRO DA PROLIFERAÇÃO DE CÉLULAS DE MELANOMA SUBMETIDAS À ALTAS DOSES DE VITAMINA D

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ABSTRACT: **Aim**: This study aimed to investigate the effects of vitamin D on the proliferation and apoptosis of melanoma cells. **Methodology**: For this purpose, the B16F10 cell line was treated with different concentrations of vitamin D for 13 hours. Then, cell viability was analyzed by the MTT assay. Student One-Way Analysis of Variance (ANOVA), with Tukey's post-hoc test, was used to evaluate significant variations between the data sets. **Results**: The concentration 10 times higher than the physiological one showed the most pronounced cytotoxic effect, followed by concentrations of 20 and 1 time. Vitamin D overload led to a decrease in the viability of B16F10 cells. **Conclusions**: The different concentrations of vitamin D used in this study (200 ng/mL and 400 ng/mL) led to a decrease in the viability of the murine melanoma cell line, while the cytotoxic effect was observed through the reduction in absorbance presented by the MTT method.

KEYWORDS: Melanoma. Vitamin D. Ultraviolet Rays.

RESUMO: **Objetivo**: Este estudo teve como objetivo investigar os efeitos da vitamina D na proliferação e apoptose de células de melanoma. **Metodologia**: Para isso, a linhagem B16F10 foi tratada com diferentes concentrações de vitamina D por 13 horas. Em seguida, a viabilidade celular foi analisada pelo ensaio MTT. A análise de variância Student One-Way (ANOVA), com o teste post-hoc de Tukey, foi utilizada para avaliar as variações significativas entre os conjuntos de dados. **Resultados**: A concentração 10 vezes maior que a fisiológica mostrou o efeito citotóxico mais pronunciado, seguida pelas concentrações de 20 e 1 vez. A sobrecarga de vitamina D levou à diminuição da viabilidade das células B16F10. **Conclusões**: As diferentes concentrações de vitamina D utilizadas neste estudo (200 ng/mL e 400 ng/mL) levaram a uma diminuição na viabilidade da linhagem celular de melanoma murino, já o efeito citotóxico foi observado através da redução da absorbância apresentada pelo método MTT.

PALAVRAS-CHAVE: Melanoma. Vitamina D. Raios Ultravioleta.

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INTRODUCTION

Skin cancer development is influenced by genetic and environmental factors. Certain hereditary components, including gene mutations, skin type, hair and eye pigmentation, and deficiencies in DNA repair mechanisms, can predispose individuals to skin cancer[1]. Environmentally, ultraviolet radiation (UVR) is a significant risk factor. Chronic or cumulative exposure to UVR disrupts melanogenesis, inducing an excitatory state in melanocytes that can become neoplastic[2,3]. UVR compromises DNA repair mechanisms, causes immunosuppression, and generates reactive oxygen species (ROS), all of which contribute to photocarcinogenesis[4].

Despite its harmful effects, UVR exposure also has beneficial outcomes, such as the synthesis of vitamin D. This liposoluble isoprenoid hormone precursor undergoes a photochemical reaction in the skin mediated by UVR, producing vitamin D3 (cholecalciferol). Vitamin D3 is biologically inactive until it is enzymatically converted in the liver and kidneys into 1,25-dihydroxycholecalciferol, a hormone that regulates calcium metabolism in the kidneys, intestines, and bones[5,6]. Importantly, vitamin D has demonstrated anticancer properties due to its ability to inhibit neoplastic proliferative processes[7,8,9].

Vitamin D deficiency has been associated with an increased risk of developing various inflammatory and chronic diseases, including asthma, infectious diseases, and certain cancers. Studies have shown that higher levels of vitamin D are correlated with a reduced incidence of some cancers, suggesting its role as a prophylactic factor and its potential to offer a good prognosis[5]. In vitro studies have indicated that vitamin D exerts antiproliferative effects on neoplastic cells, inducing pro-apoptotic and antiproliferative genes in cancers such as prostate, breast, colon, and leukemia[32].

About clinical trials, the results suggested that improving vitamin D status through increased exposure to sunlight and vitamin D supplement might reduce prostate cancer risk[10]. In another study, high vitamin D levels significantly improved clinical outcomes in Brazilian postmenopausal women with breast cancer[11]. Epidemiological studies conducted on colorectal cancer provided strong support for inverse association between serum 25(OH)D level and colon cancer risk in both men and women[12].

Clinical studies also have observed that patients with melanoma often have reduced serum levels of vitamin D3. This has led to suggestions that high-dose vitamin D supplementation following primary tumor removal may be beneficial for both the prevention and treatment of melanoma[13,14]. Additionally, in vitro studies have demonstrated that vitamin D can modulate oxidative stress in A375 cells[15] and inhibits the growth of myeloma cells in a PTEN and vitamin D receptor-dependent (VDR) manner[16].

While the potential benefits of vitamin D in cancer prevention are promising, the evidence is not yet conclusive. Researchers are still investigating the optimal levels of vitamin D for cancer prevention and whether vitamin D supplementation can improve outcomes for patients with cancer. High doses of vitamin D, for example, are being studied in clinical trials to determine their effects on slowing cancer progression or enhancing the efficacy of cancer treatments[3].

In the context of health promotion, raising awareness about the importance of vitamin D and encouraging safe sun exposure, as well as the use of supplements, when necessary, is vital. However, individuals should consult healthcare professionals before taking high doses of vitamin D, as excessive intake can lead to health complications[4].

Based on this information, the present study aimed to investigate the effects of vitamin D treatment on the proliferation of melanoma cells. For this, B16F10 cell lineage was treated with different concentrations of vitamin D (1000 IU), and the MTT assay (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was used to evaluate cytotoxic effects.

METHODOLOGY

CELL CULTURE

Murine melanoma B16F10 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and a 1% penicillin/streptomycin antibiotic mixture at a concentration of 100 µg/mL. A controlled environment in a humidified incubator with 5% CO_2 at 37°C was maintained throughout the cell culture process.

MTT ASSAY

To examine the cytotoxicity effects of vitamin D on B16F10, the MTT method was chosen. The cell cultures were seeded in a 96-well plate at $5x10^4$ cells/well and incubated for 24 hours in 5% CO_2 at 37°C. Following the incubation, the cells were treated with vitamin D (1000 IU; obtained from commercial compounding pharmacy) at concentrations of 20, 200 and 400 ng/mL for 13 hours. Subsequently, 10 µL of 5 mg/mL MTT reagent was added to each well and further incubated for 4 hours in a humidified incubator with 5% CO_2 at 37°C. The plates were observed under a microscope to demonstrate the formation of formazan crystals. After aspirating the cell culture media containing MTT, 100 µL of isopropyl alcohol was added. Absorbances were measured at 590 nm using spectrophotometer Reader Synergy H1 Hybrid Reader (Biotek).

STATISTICAL ANALYSIS

Statistical analyses were done using PRISM 4 software. Student's One-Way analysis of variance (ANOVA) with post-hoc Tukey (Honestly Significant Difference) test was used to evaluate the significance variations among data sets. In this study, $p < 0,001$ was considered statistically significant.

RESULTS

The cell viability assay conducted using the MTT method revealed that all tested concentrations of vitamin D induced significant cytotoxicity relative to the control group, which was normalized to 100% viability. Notably, the concentration of vitamin D at 10 times the physiological level elicited the most pronounced cytotoxic effect. This was followed by the concentrations at 20 and 1 time the physiological level (Figure 1).

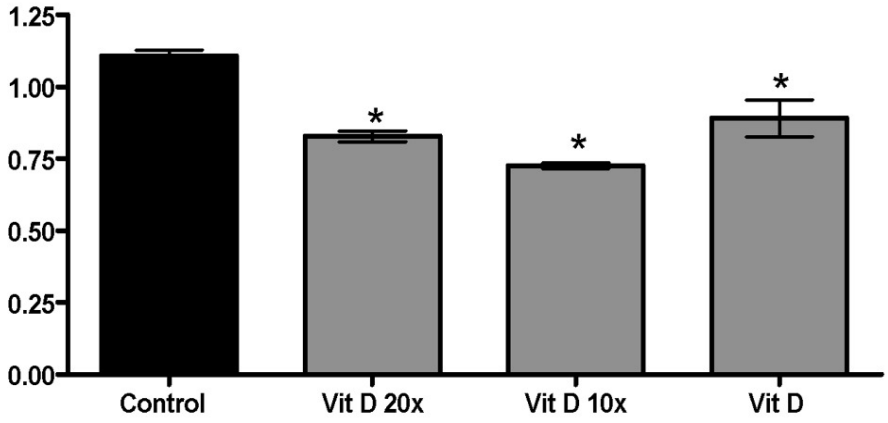

Figure 1. Analysis of the proliferation of B16F10 cells treated with high doses of vitamin D by MTT assay. Absorbances were measured at 590 nm using a spectrophotometer. Student's One-Way analysis of variance (ANOVA) with post-hoc Tukey test was used to evaluate the significance variations. *p < 0,001 was considered statistically significant.
Source: the authors.

DISCUSSION

Currently, there is a constant effort to find effective treatments and prophylactic measures for cancer progression. The challenge of providing comfort and security to patients through non-invasive and easily accessible treatments remains a global issue[17,18]. Over the past few decades, numerous authors have suggested the potential use of vitamin D overload for treatment, therapeutic stimulation, and containment of neoplastic progression[19,20,21,22].

In a review article by Brożyna and collaborators (2020)[8], it was demonstrated that high serum levels of vitamin D are related to a lower incidence of metastasis. Vitamin D and its byproducts can inhibit cell proliferation, a phenomenon observed in our study. We found that increasing the treatment concentration resulted in a decrease in the cell line concentration. This phenomenon can be explained by several factors. As cited by Brożyna[8], it can be attributed to the increased expression of cell cycle inhibitors such as p21 and p27, signaling proteins like IGFBP3 and TGF-β, and the downregulation of the hedgehog signaling pathway. The promotion of apoptosis has also been associated with vitamin D and its byproducts through the downregulation of AKT and ERK proteins and the modulation of BCL2 family proteins, including BAX, BAK, and BAD[23,24,25,26].

In the present study, a reducing effect on the B16F10 cell line was observed. A similar study by Wang and collaborators (2020)[27] using DT20 breast cancer cells showed that the growth rate was slowed with increasing concentrations of $1,25(OH)_2VitD3$, exhibiting a toxic effect at doses above 10^{-7} M. One explanation for the impaired cell growth is the difficulty in division and survival of the cell line at high treatment concentrations, which directly leads to the production of substances involved in cell adhesion and differentiation. This process can influence cell growth through the emergence of new proteins[28,29,30].

Brożyna et al. (2020)[8] also explained this effect due to the antitumor action of the active form of vitamin D, $1,25(OH)_2VitD3$, through its direct effect on cell proliferation, apoptosis, and differentiation, which directly interferes with the cell cycle and growth of the cell line. Sutedja and collaborators (2020)[31]

pointed out that both 1,25(OH)$_2$D3 and 20(OH)D3 can inhibit cancer cell proliferation by inducing apoptosis through ROS control and the inhibition of pro-apoptotic cytokines and pro-metastatic gene expression.

The reduction in cancer cell viability was also observed in a study by Hussein (2017)[32], which evaluated the inhibition of head and neck carcinoma (JHU-29 cell line) and found a dose-dependent decrease in cell viability with 1,25(OH)$_2$D$_3$. Li et al. (2021)[33] reported the inhibitory effect of 1,25(OH)$_2$D3 on various gastric cancer cell lines (MLN45, KATO III, and MKN28). While the treatment had no effect on the MKN28 cell line, the other two cell lines showed dose-dependent viability inhibition and decreased colony formation. This can be explained by the activation of the vitamin D receptor, which also increases in a dose-dependent manner.

At concentrations of 400 ng/mL, there was an increase in the viability of the B16F10 cell line compared to 200 ng/mL, although still below the physiological concentration of adult individuals. This action can be explained by the cellular adaptation to the increased concentration of vitamin D. A similar study using the MCF-7 breast cancer cell line by Diesing and collaborators (2006)[34] demonstrated that stimulation with vitamin D3 and 25-hydroxyvitamin D3 overloads resulted in increased expression of vitamin D-metabolizing enzymes. This indicates that the cell line can regulate expression, particularly of 24-hydroxylase, providing a protective effect against the apoptosis-inducing effects of calcitriol.

The use of vitamin D for neoplastic treatment is a promising area. In addition to its ability to inhibit cell proliferation and the cell cycle, it can be combined with chemotherapy to enhance therapeutic efficacy[35]. Studies have already related its capacity to sensitize malignant cells to ionizing radiation and proton beam radiation, as well as various classes of chemotherapeutics. For instance, Maj and collaborators (2018)[36] found that adding vitamin D treatment to Imatinib and cisplatin reduced the inactivation of the vitamin D analog in tumors, increasing its availability. Similarly, Alizadeh-Navaei and collaborators (2019)[37] observed that the combined treatment of vitamin D with 5-FU and cisplatin had a high cytotoxic effect on AGS gastric cancer cell lines.

CONCLUSION

The different concentrations of vitamin D used in this study (200 ng/mL and 400 ng/mL) led to a decrease in the viability of the murine melanoma cell line. The cytotoxic effect was observed through the decreased absorbance presented by the MTT method. The potential applications of this treatment are numerous, ranging from individual use to synergistic treatment, as well as for prophylactic effects and stabilization of neoplasia, resulting in a good prognosis for the patient. However, it is important to note that our study has limitations, as it was conducted only in vitro, and further studies in animal models are needed. Another limiting factor is the high doses of vitamin D used, which may promote cytotoxic effects in animals and humans.

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