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ROLE OF VITAMIN D IN THE EFFECTIVENESS OF CHEMOTHERAPEUTIC DRUGS ON GASTRIC CANCER CELL LINES

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Abstract – *Background:* *Gastric cancer is a common cause of death worldwide. It is highly prevalent in Iran, especially in the north. It appears that vitamin D (Vit D) has a role in preventing cancer cells from proliferating. Thus, this study aimed to determine the role of Vit D in the effectiveness of chemotherapeutic drugs on cellular categories.*

Patients and Methods: *The MTT assay, flow cytometry, and TNF-α cytokine concentration measurement were employed in this experimental study to evaluate the cytotoxicity effects of Vit D on cellular categories of gastric cancer such as MKN 45 (NC: C615), KATO III (NC: C640), and AGS (NC: C131). The statistical t-test was utilized in SPSS 16 to analyze data and determine the mean of 24 h cell proliferation and cytokine generation.*

Results: *This study investigated the toxicity effect using the MTT assay based on combined treatment with Vit D and chemotherapeutic drugs. The results indicated a statistically significant difference ($p<0.05$) between cancer cells and normal cells in the base dose (μM50). Regarding the tested cellular categories, AGS and KATO III showed the highest (83%, 57%) and lowest (2%, 38%) cytotoxicity means of combined interventions (Vit D with chemotherapeutic drugs), respectively. Moreover, despite the decreased cell viability in different concentrations of Vit D, this decrease was not statistically significant ($p>0.05$). According to the flow cytometry results, the apoptosis percentages of 5FU group (60.7%) and 5FU-Vit D group (76.05%) were higher than that of the control group (25.66%).*

Conclusions: According to the research results, Vit D decreased the viability of gastric cancer cells and infused the apoptosis process into them. It also prevented the proliferation of cancer cells, something which can be used in the future to treat patients after conducting animal studies and clinical trials.

KEYWORDS: *Vit D, Gastric cancer, Apoptosis, MTT, Flow cytometry.*

INTRODUCTION

Gastric cancer is considered the fourth most common cancer[1] and the second cause of death from cancer worldwide[1]. The prevalence of gastric cancer is very high in Asia[3]. In Iran, it is the third cause of death and the north and northwest of Iran are among the highest-risk areas of gastric cancer[4,5]. The most important risk factors of this disease include positive family history, certain diets, premalignant lesions of the stomach, genetic factors, geographical factors, and tobacco consumption[6]. Although regarded as the main treatments for gastric cancer, surgery and chemotherapy are very prone to relapse and damage

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the normal tissues[7]. In recent years, many studies have been conducted on the hypothesis about the relationship between serum levels of Vitamin D (Vit D) and the risks of various cancers[8,9]. Several studies show that Vit D plays a key role in cell proliferation, differentiation and termination of the cell cycle, and the inhibition of metastasis[10,11]. It also has a role in the prognosis of patients with gastric cancer by indicating the relationship between Vit D deprivation and metastasis resulting in the low survival rate of patients[12]. Given the effects of genetic and geographical factors along with different treatments and contradictory theories regarding the role of Vit D in the treatment of gastric cancer, this study aimed to determine the role of Vit D in the effectiveness of chemotherapeutic drugs on gastric cancer cell lines.

MATERIALS AND METHODS

The MTT assay, flow cytometry, and TNF-α cytokine concentration measurement were employed in this experimental study to determine the cytotoxic effects of Vit D on HGF3-PI53 cellular categories of normal gingival cells of human origin (NCBI Code: C502) and certain cellular categories of gastric cancer such as MKN 45 (NC: C615), KATO III (NC: C640), and AGS (NC: C131). The goal was to determine the role of Vit D in the effectiveness of chemotherapeutic drugs on cellular categories.

Cell Culture

HGF3-PI53 cellular categories of normal gingival cells of human origin (NCBI Code: C502) and certain cellular categories of gastric cancer such as MKN 45 (NC: C615), KATO III (NC: C640), and AGS (NC: C131) were purchased from the Pasteur Institute of Iran (PII) in frozen vials. These cellular categories were cultured in RPMI-1640 containing 5-10% of FBS (Fetal Bovine Serum), 100 U/ml of penicillin, and 100 µg/ml of streptomycin. The cell-containing flask was put into an incubator running at 37ºC with a 5% pressure of CO2 and 95% of humidity. Dose and time were evaluated to determine the optimal effects of synthesized Vit D3. These cells were treated with 0, 50, 75, 100, 125, 200, 300, and 400 micromolar concentrations of Vit D3 in an intervention combined with cisplatin and 5-FU. They were then analyzed after 24 h. The zero concentration was used in the control group. The tests were triplicated to increase the comparison accuracy and enhance the process efficiency. Each of the concentrations was separately poured on plate pits to control the optical absorption of the drug.

Vitamin D3 Cytotoxicity

The MTT colorimetric test was conducted to determine the cytotoxicity of synthesized Vit D3 on all of the cellular categories. The MTT powder is a yellow tetrazolium water-soluble salt, which is recovered by succinate dehydrogenase enzymes existing in the mitochondria of living cells and transformed into the water-insoluble formazan color. In brief, 5×103 cells were cultured in each plate with 96 cells. After an overnight sleep in the new environment, the cells were treated with different doses of Vit D for 24 h. Then, the MTT solution (5 mg/ml in PBS) was added to plate pits which were then kept in an incubator for 3 h. As the next step, the supernatant solution was extracted, and 200 ml of Dimethyl Sulfoxide (DMSO) was added to each pit to break the cell membrane and allow the formazan colors to exit. For this purpose, the plate was put on a shaker for 20 min. Finally, the samples were measured by a microplate reader in a wavelength of 570 nm with a reference of 630. GraphPad Prism 6 was employed to determine the value of IC50 (indicating a concentration of a drug inhibiting 50% of cell growth) in different cellular categories.

Apoptosis by Flow Cytometry

The cells were colored with Annexin-V and PI by using the Annexin V-FITC Apoptosis Detection Kit (eBioscience, US, cat. no: BMS500FI-20) to determine the percentage of apoptosis cells out of the cells treated with the proposed intervention and compare it with the population of the negative control cells. After preparing AGS cells and exposing them to the proposed intervention for 24 h, the cell sediment was washed by adding 1 ml of PBS in a centrifuge running at 1800 rpm for 5 min. After that, the resultant sediment was rewashed and homogenized with a binding buffer solution. To add colors, 5 µl of Annexin was first added to the relevant tubes incubated at the room temperature for 5 min. After washing it with the buffer, 10 µl of PI was added to the resultant sediment incubated in a dark environment at the room temperature for 20 min. In the last step, the final volume of each tube was increased to 1 ml by using the binding buffer then, the cell analysis was performed by the FACscan device (Partec, PAS), in which the data analysis were conducted by a built-in program, too. The cells were located on 4 quarters of the output curve: Q1 indicated the necrosis cells (PI+, Annexin-V+); Q2 showed the late apoptosis (PI-, Annexin-V+); Q3 depicted healthy cells (PI+, Annexin-V+); and Q4 presented the early apoptosis (PI-, Annexin-V-).

TNF-a Cytokine Concentration

TNF-α cytokine was evaluated in the supernatant liquid resulting from the cultivation of AGS cells treated with a 200 µM concentration of Vit D3, a 20 µM concentration of 5-FU, and a mixture of these interventions. This measurement was performed using an ELISA assay conducted by the human

TABLE 1. The inhibition percentage mean of Vit D3 in combination with chemotherapeutic drugs for different cellular categories (mean ± SD).

Cell lines/Intervention	*AGS*	*HGF*	*MKN 45*	*KATO III*
D3 (50µM)	0.06±0.17	0.15±0.15	0.07±0.09	0.11±0.00
D3 + 5-FU	0.43 ± 0.03	0.61 ± 0.02	0.46 ± 0.03	0.98 ± 0.04
***p*-value**	0.05	0.05	0.00	0.02
D3 + Cisplatin	0.17 ± 0.01	0.28 ± 0.03	0.35 ±0.03	0.62 ± 0.09
***p*-value**	0.02	0.00	0.00	0.00

TNF-α concentration measurement kit powered by Bt-Laboratory (cat no. E0082HU, China, Sensitivity; 1.52 ng/L) in accordance with the manufacturer instructions. The samples were evaluated 3 times to determine the absorption mean of each sample.

Statistical Analysis

Data analysis was carried out in SPSS 16 (SPSS Inc., SPSS for Windows, Chicago, IL, USA). After checking the normality of the distribution of variables, the *t*-test was employed to determine the means of cell proliferation and cytokine generation. The growth inhibition and cytokine concentration were reported in mean ± standard deviation, and $p<0.05$ was regarded as the significance level. Then, GraphPad Prism was utilized to determine IC50, and the diagrams were plotted in Excel 2007.

RESULTS

Analyzing the Effects of D3 Cytotoxicity

The cytotoxicity effect was determined by a combined treatment of Vit D and chemotherapeutic drugs. Accordingly, there was a statistically significant difference ($p<0.05$) between cancer cells and normal cells in the base dose (50 µM). The cytotoxicity means of combined interventions (Vit D with chemotherapeutic drugs such as 5FU and cisplatin) were the highest (83%, 57%) for the AGS cellular category and the lowest (2%, 38%) for the KATO III cellular category. Therefore, the latter was selected as the appropriate category for apoptosis determination and the reduction of inflammatory secretions (Table 1). According to Figure 1, the viability of cells decreased in exposure to different concentrations of Vit D. However, there was no statistically significant decrease ($p>0.05$) in comparison with the base concentration (50 µM). Table 2 shows the amounts of IC50 determined for each cellular category.

Figure 1 shows the viability results of treating AGS, MKN 45, KATO III, and HGF cells with the mentioned concentrations of Vit D3 for 24 h. According to this figure, the proposed intervention indicated no absorption in any doses.

Apoptosis Evaluation with the Annexin Test

Figure 2 shows the Annexin test results in AGS cells treated with Vit D3 (200 µM), 5-FU (20 µM), and the combination of the two interven-

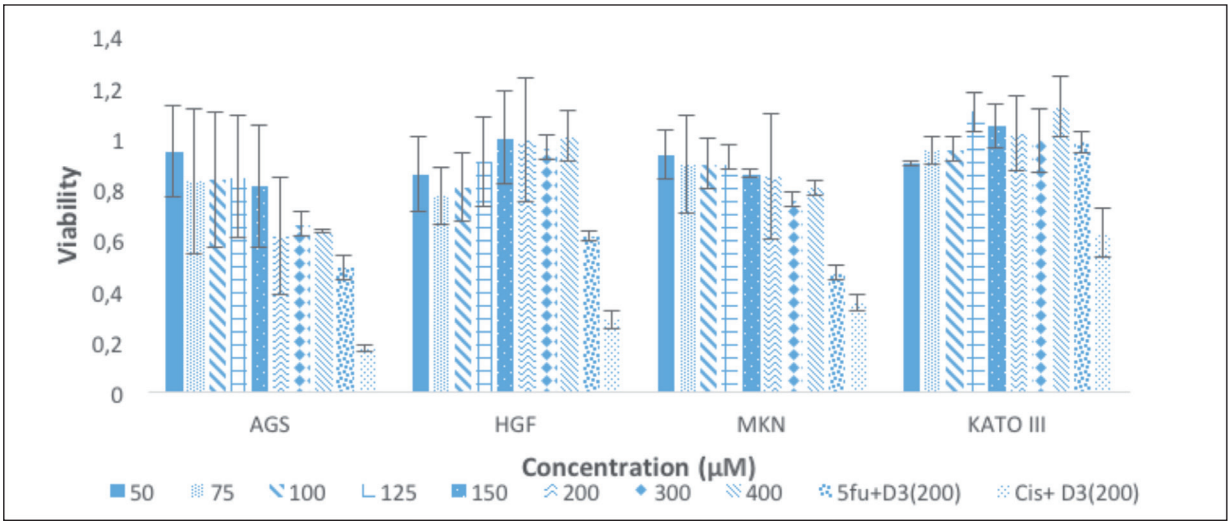

Fig. 1. The viability effects of Vit D3 on AGS, MKN 45, KATO III, and HGF Cells for 24 h.

TABLE 2. IC50 of Vit D3 on AGS, MKN 45, KATO III, and HGF Cells for 24 h.

IC50	
MKN 45	215.7 µM
KATO III	245.6 µM
HGF	239.2 µM
AGS	230 µM

tions after analyzing the cells colored with PI and Annexin-V through flow cytometry in Flow-Max. The data analysis was done by a built-in program. The resultant points were then plotted on a 2D diagram divided into four quarters (Q). According to the research findings, the amount of apoptosis cells (early, late, and necrotic) was 25.66% in the control group cells (Quarter D), 29.59% in the Vit D group cells with a concentration of 200 µM (Quarter A), 60.71% in the 5-FU group cells with a concentration of 20 µg (Quarter B), and 76.05% in the combined group cells (5-FU and Vit D) (Quarter C).

TNF-α Cytokine Secretions

The results of data obtained from the concentrations of cytokine secreted by AGS cells were analyzed in three intervention groups. Accordingly, the TNF-α secretions decreased in groups treated with the proposed interventions in comparison with that of the untreated group. However, there was no statistically significant difference (p>0.05) (Table 3).

DISCUSSION

Regarded as an anticancer agent, Vit D shows different biological activities such as the genetic adjustment of cells, apoptosis infusion, and cell cycle inhibition[10,11]. According to the epidemic studies[11,13,14], the deprivation of Vit D3 serum level can increase the risk of colorectal, breast, and prostate cancers. Furthermore, the lack of Vit D is related to metastasis and the short-term survival of patients with gastric cancer[12]. In this study, the MTT assay was employed along with the Vit D treatment combined with chemotherapeutic drugs to show that there was a significant relationship between cancer cells and normal cells in the cytotoxic effects. The results were consistent with the findings of Baek et al[11]. However, there was no statistically significant difference in the viability of cells exposed to different concentrations of Vit D, a finding which is inconsistent with those of Baek et al[11] and Park et al[15]. During the apoptosis process, phosphatidylserine leaked from the inner cell membrane to the outer cell membrane, and the Annexin-V joined the phosphatidylserine existing in the outer cell membrane. Moreover, PI joined the fragmented pieces of the core DNAs of apoptosis cells, which could be

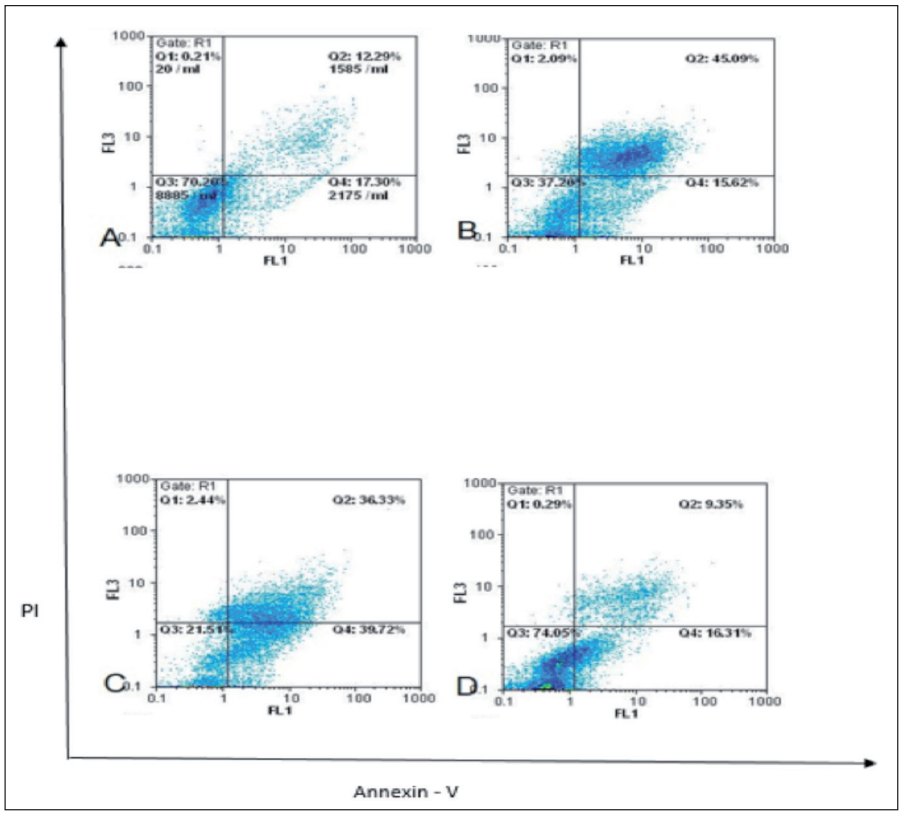

Fig. 2. The flow cytometry results of AGS cells: control group (D); treated with the anticancer drug 5-FU (B); Vit D combined with 5-FU (C); and Vit D3 (A).

TABLE 3. TNF-α secretions (ng/L) in different interventions applied to AGS cells.

Intervention	*D3*	*5-FU*	*D3 + 5-FU*	*Untreated*
Concentration	210±68	232±104	189±100	243±52
p-value	0.556	0.882	0.456	

detected by the flow cytometry device[16]. According to the apoptosis analysis, the apoptosis percentage (Q2 and Q4) of 5-FU (B) and 5FU-Vit D group (C) was higher than that of the control group, a finding which was consistent with that of Pan et al[7]. According to Kemnitzer et al[17], the stimulation of receptors such as the tumor necrosis factor (TNF) at a cellular level could infuse the apoptosis process through an external passage, a finding which was inconsistent with those of the present study. The results of this study showed that Vit D decreased the viability of gastric cancer cells, infused the apoptosis process into these cells, and prevented them from proliferating. Considering the budget constraints on this study, it is recommended to employ more sensitive kits in the future studies to analyze the concentration of cytokine with the ELISA assay.

Conflict of Interest

The Authors declare that they have no conflict of interests.

REFERENCES

1) Jemal A, Center MM, DeSantis C, Ward EM. Global patterns of cancer incidence and mortality rates and trends. Cancer Epidemiol Biomarkers Prev 2010; 19:1893-1907.
2) Zhang H, Jin G, Li H, Ren C, Ding Y, Zhang Q, Deng B, Wang J, Hu Z, Xu Y, Shen H. Genetic variants at 1q22 and 10q23 reproducibly associated with gastric cancer susceptibility in a Chinese population. Carcinogenesis 2011; 32: 848-852.
3) Fock KM, Ang TL. Epidemiology of helicobacter pylori infection and gastric cancer in Asia. J Gastroenterol Hepatol 2010; 25: 479-486.
4) Mohebbi M, Mahmoodi M, Wolfe R, Nourijelyani K, Mohammad K, Zeraati H, Fotouhi A. Geographical spread of gastrointestinal tract cancer incidence in the Caspian Sea region of Iran: spatial analysis of cancer registry data. BMC Cancer 2008; 8: 137.
5) Hartgrink HH, Jansen EP, van Grieken NC, van de Velde CJ. Gastric cancer. Lancet 2009; 374: 477-490.
6) Keyhanian SH, Farhadifar N, Fotoukian Z, Pouya M, Saravi M. Epidemiologic and malignancy indices of gastric cancer in patients referred to oncology clinic at ramsar emam sajjad hospital during 2002-2009. Journal of Shahid Sadoughi University of Medical Sciences 2012; 20: 110-118.
7) Pan L, Matloob AF, Du J, Pan H, Dong Z, Zhao J, Feng Y, Zhong Y, Huang B, Lu J. Vitamin D stimulates apoptosis in gastric cancer cells in synergy with trichostatin A/sodium butyrate-induced and 5-aza-2¢-deoxycytidine-induced PTEN upregulation. FEBS J 2010; 277: 989-999.
8) Abnet CC, Chen Y, Chow WH, Gao YT, Helzlsouer KJ, Le Marchand L, McCullough ML, Shikany JM, Virtamo J, Weinstein SJ, Xiang YB, Yu K, Zheng W, Albanes D, Arslan AA, Campbell DS, Campbell PT, Hayes RB, Horst RL, Kolonel LN, Nomura AM, Purdue MP, Snyder K, Shu XO. Circulating 25-hydroxyvitamin D and risk of esophageal and gastric cancer: Cohort Consortium Vitamin D Pooling Project of Rarer Cancers. Am J Epidemiol 2010; 172: 94-106.
9) Jamshidinaeini Y, Akbari ME, Abdollahi M, Ajami M, Davoodi SH. Vitamin D status and risk of breast cancer in Iranian women: a case-control study. J Am Coll Nutr 2016; 35: 639-646.
10) Heaney RP. The Vitamin D requirement in health and disease. J Steroid Biochem Mol Biol 2005; 97: 13-19.
11) Baek S, Lee YS, Shim HE, Yoon S, Baek SY, Kim BS, Oh SO. Vitamin D3 regulates cell viability in gastric cancer and cholangiocarcinoma. Anat Cell Biol 2011; 44: 204-209.
12) Ren C, Qiu MZ, Wang DS, Luo HY, Zhang DS, Wang ZQ, Wang FH, Li YH, Zhou ZW, Xu RH. Prognostic effects of 25-hydroxyvitamin D levels in gastric cancer. J Transl Med 2012; 10: 16.
13) Bertone-Johnson ER, Chen WY, Holick MF, Hollis BW, Colditz GA, Willett WC, Hankinson SE. Plasma 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D and risk of breast cancer. Cancer Epidemiol Biomarkers Prev 2005; 14: 1991-1997.
14) Ahonen MH, Tenkanen L, Teppo L, Hakama M, Tuohimaa P. Prostate cancer risk and prediagnostic serum 25-hydroxyvitamin D levels (Finland). Cancer Causes Control 2000; 11: 847-852.
15) Park MR, Lee JH, Park MS, Hwang JE, Shim HJ, Cho SH, Chung IJ, Bae WK. Suppressive effect of 19-nor-1α-25-dihydroxyvitamin D2 on gastric cancer cells and peritoneal metastasis model. J Korean Med Sci 2012; 27: 1037-1043.
16) Hosseini J, Mahmoodi M, Hakhamaneshi MS, Jalili A, Khoshdel AR, Sheikhfathollahi M, Fakhari SH, Hosseini-Zijoud SM. Apoptosis effects of aloe-emodin against MCF-7 cell line. J Rafsanjan Univ Med Sci 2013; 13: 41-52.
17) Kemnitzer W, Kasibhatla S, Jiang S, Zhang H, Zhao J, Jia S, Xu L, Crogan-Grundy C, Denis R, Barriault N, Vaillancourt L, Charron S, Dodd J, Attardo G, Labrecque D, Lamothe S, Gourdeau H, Tseng B, Drewe J, Cai SX. Discovery of 4-aryl-4 H-chromenes as a new series of apoptosis inducers using a cell- and caspase-based high-throughput screening assay. 2. Structure-activity relationships of the 7- and 5-, 6-, 8-positions. Bioorg Med Chem Lett 2005; 15: 4745-4751.