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RESEARCH

Enhancing effect of bevacizumab in the classic bleomycin, etoposide, cisplatin treatment applied in human testicular germ cell tumors

İnsan testis germ hücreli tümörlerinde uygulanan klasik bleomisin, etoposid, sisplatin tedavisinin bevacizumab ile güçlendirilmesinin etkisi

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Abstract

Purpose: This study aimed to evaluate the therapeutic potential of Bevacizumab, a vascular endothelial growth factor (VEGF) inhibitor, in combination with the standard Bleomycin, Etoposide, and Cisplatin (BEP) regimen for the treatment of testicular germ cell tumors (TGCTs). The primary focus was to investigate its ability to enhance treatment efficacy while reducing cytotoxic side effects.

Materials and Methods: The half-maximal inhibitory concentration (IC_{50}) of the BEP regimen was determined in the 1618-K human TGCT cell line and subsequently combined with varying concentrations of Bevacizumab. Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Expression levels of VEGF, Bax, Bcl-2, and Caspase-3 were analyzed by immunohistochemistry.

Results: Bevacizumab significantly reduced cell viability in a dose-dependent manner. Bax expression in Group 2 (2.05 ± 1.04) was significantly lower than in the control group (8.63 ± 2.57;). Caspase-3 expression in Group 2 (0.49 ± 0.08) was markedly decreased compared with the control (5.10 ± 3.82), and a similar reduction was also observed in Group 3. Despite the absence of substantial changes in apoptotic markers, the reduction in cell viability suggests that Bevacizumab may act through alternative mechanisms.

Conclusion: By targeting tumor vascularity, Bevacizumab may enhance the therapeutic efficacy of the BEP regimen in TGCT treatment. These findings support the potential role of Bevacizumab as an adjunctive agent in TGCT therapy and highlight the need for further validation through in vivo studies.

Keywords:. Testicular germ cell tumors, bevacizumab, VEGF, cell culture techniques

Öz

Amaç: Bu çalışmanın amacı, testiküler germ hücreli tümörlerin (GCTT) tedavisinde yaygın olarak kullanılan Bleomisin, Etoposid ve Sisplatin (BEP) kombinasyon tedavisinin etkinliğini artırmak ve sitotoksik yan etkilerini azaltmak üzere vasküler endotelyal büyüme faktörü (VEGF) inhibitörü olan Bevacizumab'ın terapötik potansiyelini değerlendirmektir.

Gereç ve Yöntem: 1618-K insan GCTT hücre hattında BEP tedavisinin yarı maksimal inhibitör konsantrasyonu (IC_{50}) belirlendi. Bu doz, farklı konsantrasyonlardaki Bevacizumab ile birlikte uygulandı. Hücre canlılığı 3-(4,5-dimetiltiyazol-2-il)-2,5-difeniltetrazolyum bromür (MTT) testi ile değerlendirildi. VEGF, Bax, Bcl-2 ve Kaspaz-3'ün ekspresyon düzeyleri immünohistokimya tekniği ile incelendi.

Bulgular: Bevacizumab, doz bağımlı olarak hücre canlılığını anlamlı şekilde azaltmıştır. A Bax ekspresyonu Grup 2'de (2,05±1,04) kontrol grubuna (8,63±2,57) göre anlamlı şekilde azalmıştır. Caspase-3 düzeyi Grup 2'de (0,49±0,08) kontrol grubuna (5,10±3,82) göre anlamlı olarak azalmış, Grup 3'te de benzer bir azalma görülmüştür. Apoptotik belirteçlerde anlamlı değişiklik olmamasına rağmen, hücre canlılığındaki azalma Bevacizumab'ın farklı mekanizmalarla etkili olabileceğini göstermektedir.

Sonuç: Bevacizumab'ın tümör vaskülaritesini hedef alarak BEP tedavisinin etkinliğini artırabileceği düşünülmektedir. Bu bulgular, Bevacizumab'ın GCTT tedavisinde destekleyici bir ajan olarak kullanılma potansiyeline işaret etmekte ve in vivo çalışmalarla desteklenmesi gerektiğini göstermektedir.

Anahtar kelimeler: Testiküler germ hücreli tümörler, bevacizumab, VEGF, hücre kültürü teknikleri

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INTRODUCTION

Germ cell tumors of the testis (GCTT) represent the most common malignancy in young men worldwide, and their incidence is increasing. Approximately 95% of these tumors originate from germinal tissue[1,2]. In the treatment of testicular germ cell tumors, either a dual-drug or triple-drug regimen is applied. The most commonly used combination is the triple-drug regimen consisting of Bleomycin, Etoposide, and Cisplatin (BEP)[3]. Conventional BEP treatment in GCTT has been shown to increase patient survival rates to approximately 90%. However, the three drugs used in BEP therapy are associated with significant side effects[4]. Among these, bleomycin-induced pneumonitis is the most severe and potentially fatal. Pulmonary changes following BEP therapy have been demonstrated using computed tomography. Additionally, cisplatin, one of the agents included in the BEP protocol, has been associated with significant toxicities such as tubular nephropathy and hearing loss[5].

For this reason, we hypothesized that an additional drug that could enhance the efficacy of the triple therapy while reducing side effects might be beneficial. In searching for potential adjunctive agents with fewer toxic effects, we considered Bevacizumab, which is an anti-cancer drug that has gained widespread use in recent years. Bevacizumab is an anti-tumor agent used in the treatment of various tumor types, targeting the blood vessels that nourish tumor cells rather than directly affecting the tumor cells themselves. It blocks vascular endothelial growth factor (VEGF), a key factor in neoplastic angiogenesis that stimulates the formation of new blood vessels[6,7]. Recent studies have also evaluated the efficacy of Bevacizumab in the treatment of metastatic colorectal cancer and its potential to extend survival times when added to chemotherapy, demonstrating that this treatment approach is beneficial[8].

Recent studies have examined the role of Bevacizumab in the treatment of metastatic colorectal cancer (mCRC) and its potential to increase extracellular matrix (ECM) accumulation associated with treatment. It has been shown that even at low doses, Bevacizumab suppresses tumor growth by 50%[9]. Additionally, treatment with Bevacizumab in combination with atezolizumab has been demonstrated to improve treatment response in hepatocellular carcinoma (HCC) patients by activating immune cells in the tumor microenvironment, particularly by enhancing CD8 effector-memory T cells and activating CXCL10+ macrophages[10].

In our study, we hypothesize that adding an additional therapeutic agent could enhance the efficacy of BEP therapy used in the treatment of GCTT while reducing its side effects. In this context, we considered Bevacizumab which has been increasingly used in recent years. We propose that Bevacizumab, by targeting the blood vessels that nourish tumor cells rather than directly affecting the tumor cells themselves, could improve the effectiveness of BEP therapy and reduce treatment-related side effects. This study aims to investigate whether Bevacizumab, through its VEGF inhibitory effect, can potentiate BEP therapy and contribute to the literature by facilitating its application in future in vivo studies.

MATERIALS AND METHODS

Procedure

Ethical approval for this study was obtained from the Afyon Kocatepe University Human Research Ethics Committee (Date: 03.02.2017, Decision No: 2017/2, Approval No: 45). The human testicular germ cell tumor (GCTT) cell line 1618-K (ACC752), used in the study, was obtained from the Leibniz Institute DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) cell bank in Germany. As no direct human or animal participants were involved, obtaining informed consent was not required.

This study was conducted in the cell culture laboratory of the Department of Histology and Embryology at Kocatepe University, Afyokarahisar. To ensure data reliability and reproducibility, all experimental procedures were performed in accordance with institutional laboratory guidelines. Experimental work and analyses were carrid out by Murat Tosun, Murat Serkant Ünal, and Deniz Koç. All cell culture experiments were independently repeated three times, producing consşstent results. Positive and negative controls were included in all experiments. Cell viability and contamination were routinely monitored. Experimental data were stored electronically and are available upon request.

Statistical analyses were performed to enhance the reliability of the data.

In our study, the half maximal inhibitory concentration (IC_{50}) dose of the BEP combination was determined in the 1618-K (ACC752) human germ cell tumor cell line under in vitro cell culture conditions. This dose was then combined with different concentrations of Bevacizumab, and cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

While obtaining MTT results, immunocytochemical staining was performed on the same samples to evaluate the expression levels of VEGF, Caspase-3, Bax, and Bcl-2. Cell counting was performed on specimens visualized under a light microscope (Nikon E-600), using the Nikon NIS-Elements 4.2 image analysis software. In each specimen, cells were manually marked and counted on the acquired digital micrographs. A total of 500 cells were evaluated in randomly selected microscopic fields per specimen and approximately 32,000 cells were analyzed across all groups.

Subsequently, the ratios of immunopositive and immunonegative cells were calculated, and the results were subjected to statistical analysis.

Determination of experimental groups and doses

In our study, the dose levels of the BEP triple regimen and the monoclonal antibody Bevacizumab to be administered to the GCTT cell line were determined based on the results of the MTT assay[7].

The applied doses in this study were as follows: when a combination of 40 mM cisplatin, 50 mM etoposide, and 100 mM bleomycin was administered, cell viability was reduced only 20%, indicating the need for dose adjustments. To address this, various combinations at different concentrations were applied using conventional methods to achieve a 50% cell death rate in the cell line. After determining this dose, it was designated as the baseline dose and combined with different concentrations of Bavacizumab toassess cellviability. Based on these findings, four experimental groups were established as follows:

1. Cisplatin 20 µM + Etoposide 25 µM + Bleomycin 50 µM
2. Cisplatin 10 µM + Etoposide 12 µM + Bleomycin 25 µM
3. Cisplatin 4 µM + Etoposide 5 µM + Bleomycin 10 µM
4. Cisplatin 2 µM + Etoposide 2.5 µM + Bleomycin 5 µM

the dose that killed half of the cells was determined. Accordingly, since the MTT results showed 51% viability at a dose of Cisplatin 2 µM + Etoposide 2.5 µM + Bleomycin 5 µM, this dose was selected as the baseline BEP dose for the study. Initially, the doses were determined empirically, and after confirming the final dose based on the MTT results, different concentrations of Bevacizumab were applied in combination with this dose. MTT measurements were then performed, and cell viabilities were calculated. Based on these results, the experimental groups were determined.

1. Control (C): Cisplatin 2 µM + Etoposide 2.5 µM + Bleomycin 5 µM
2. Group 1(G1): (Cisplatin 2 µM + Etoposide 2.5 µM + Bleomycin 5 µM) + Bevacizumab 0.01 mg/ml
3. Group 2 (G2): (Cisplatin 2 µM + Etoposide 2.5 µM + Bleomycin 5 µM) + Bevacizumab 0.05 mg/ml
4. Group 3 (G3): (Cisplatin 2 µM + Etoposide 2.5 µM + Bleomycin 5 µM) + Bevacizumab 0.1 mg/ml

Immunohistochemical staining

Immunohistochemical analysis was performed on specimens mounted onto poly-L-lysine–coated slides. Using a hydrophobic PAP-PEN marker, two wells were created on each slide. From each cryovial, 15 µl of suspension containing approximately 1×10^6 cells per milliliter of culture medium was transferred into the wells. The slides were then air-dried at room temperature to allow cell adhesion. To increase membrane permeability, a 0.2% Tween-20 solution was applied, followed by incubation for 20 minutes. The slides were then washed twice with phosphate-buffered saline (PBS) for 3 minutes each. Endogenous peroxidase activity was blocked by incubating the slides for 10 minutes in a solution containing 3% methanol and hydrogen peroxide, followed by two additional PBS washes. Primary antibodies, each diluted 1:50, were applied to the

wells: Bax (Santa Cruz Biotechnology, sc-526), Bcl-2 (Santa Cruz Biotechnology, sc-7382), VEGF (Santa Cruz Biotechnology, sc-7269), and Caspase-3 (Abcam, ab4051). The slides were incubated overnight at 4 °C. After incubation, the slides were equilibrated to room temperature for 10 minutes and washed twice with PBS. To enhance the antigen–antibody reaction, a Primary Antibody Enhancer solution was applied for 10 minutes at room temperature, followed by two PBS washes. Next, a horseradish peroxidase (HRP) polymer solution was added, and the slides were incubated for 15 minutes at room temperature, then washed twice with PBS. For color development, 3-amino-9-ethylcarbazole (AEC) single solution was applied, and the slides were incubated for 10 minutes at room temperature. The reaction was terminated by washing the slides twice with distilled water. Counterstaining was performed with Mayer's hematoxylin for 50 seconds, followed by three rinses with distilled water. Finally, the slides were mounted with a water-based mounting medium.

Evaluation of preparations and image analysis

Three technical replicates were performed for each experimental group (n=3). The 1618-K (ACC752) germ cell tumors cell line was used. Only morphologically healthy, uncontaminated cells that had reached approximately 80% confluency were included in the study. Cells exhibing signs of contamination or abnormal morphology were excluded.

Immunohistochemically stained preparations were evaluated under a light microscope. Cells in the preparations were examined under at ×40 objective magnification, and a total of 500 cells were counted in randomly selected areas of each preparation. The ratio of immunopositive to immunonegative cells was calculated, and the resulting values were used for statistical analysis. In total, at least 32,000 cells were counted across all preparations. Image analysis was performed using a Nikon E-600 light microscope, and immunopositive and immunonegative cells were quantified with the Nikon NIS-Elements 4.2 ımage analysis software.

Statistical analysis

Cell viability was assessed using the MTT assay. Statistical analyses were performed with SPSS for Windows, version 20.0 (IBM Corp., Armonk, NY, USA). Optical density data obtained for each group, as well as changes in the ratio of immunopositive to immunonegative cell counts, were summarized using descriptive statistics, including mean, standard deviation, and median values. To assess statistically significant differences between the groups, the Kruskal–Wallis test was applied. When significant differences were detected, the Bonferroni-corrected Dunn's multiple comparison test was performed to identify the specific group or groups responsible for the difference. A p-value of less than 0.05 was considered statistically significant.

RESULTS

When the findings were evaluated collectively, it was determined that Bevacizumab significantly reduced cell viability in the tumor cell line; however, this effect did not appear to be mediated through the apoptotic markers assessed in this study (Bax, Bcl-2, and Caspase-3). Bevacizumab, a VEGF inhibitor, exhibited a potential anti-invasive effect by suppressing VEGF expression at lower doses; nevertheless, this effect was diminished at higher doses, suggesting that it may have limited therapeutic applicability in GCTT. Although no significant changes were observed in the expression of the evaluated apoptotic markers, the observed reduction in viability and the increase in cell death within the same cell lines suggest that Bevacizumab may induce cell death through alternative molecular pathways.

MTT Test Results

The MTT assay demonstrated a dose-dependent decrease in cell viability with increasing concentrations of Bevacizumab (Figure 1).

Immunohistochemical staining analysis

Bax expression

Bax expression decreased with increasing doses of Bevacizumab. A statistically significant reduction was observed between the Control, G2, and G3 groups ($p < 0.05$), while no significant difference was detected between the Control and G1 groups ($p > 0.05$). Although a decrease in expression was noted in G1 and G2 compared to the Control group, a slight increase was observed in G3 relative to G2. However, this increase was not considered meaningful, as no statistically significant difference was found between G2 and G3 ($p > 0.05$) (Figure 2, Table 1).

Bcl-2 expression

Bcl-2 expression was found to be very low in all groups, including the Control group. No significant differences were observed among the groups ($p > 0.05$). Therefore, it was concluded that Bcl-2 is not expressed in this cell line, and thus does not contribute to the induction of cell death (Figure 3, Table 1).

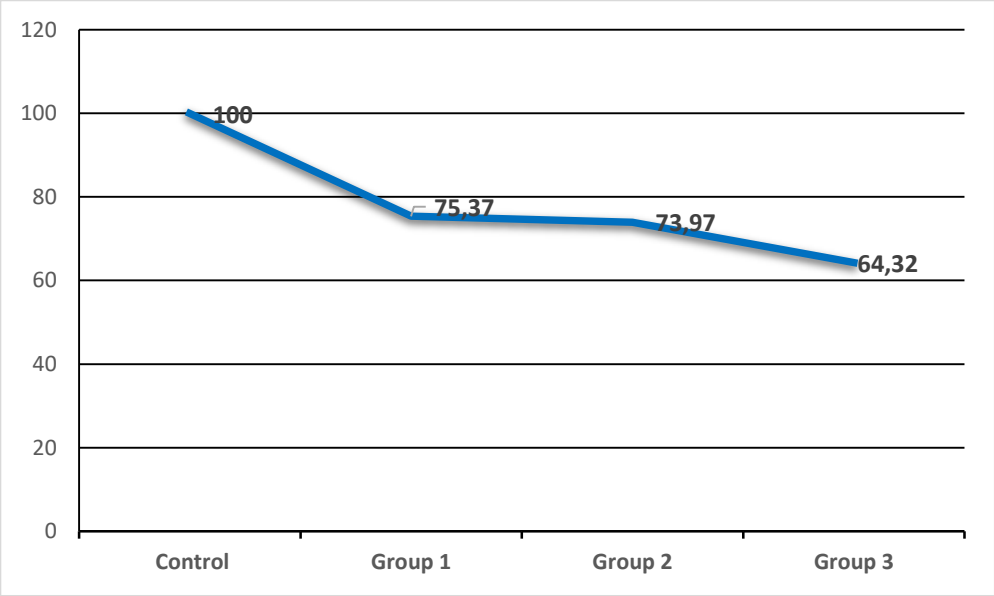

Figure 1. Cell Viability (%) in GCTT Cells After Bevacizumab Treatment (MTT Assay). (Source: Present study).

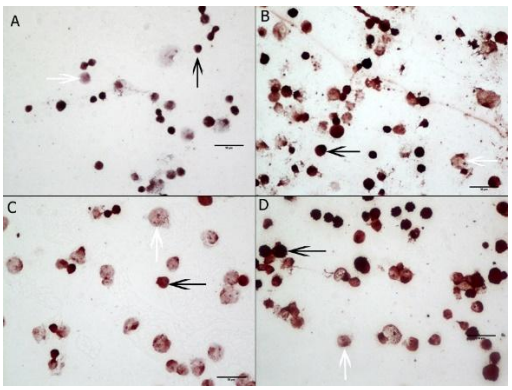

Figure 2. A (Control group), B (Group 1), C (Group 2), D (Group 3) Bax protein expression in immunopositive and immunonegative cells at ×20 magnification. (White arrow: immunonegative cell, black arrow: immunopositive cell). (Source: Present study).

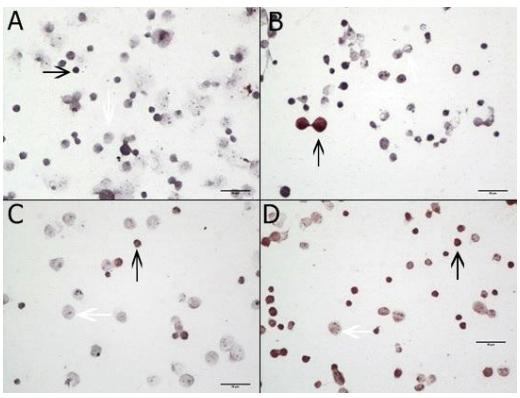

Figure 3. A (Control group), B (Group 1), C (Group 2), D (Group 3) Bcl-2 protein expression in immunopositive and immunonegative cells at ×20 magnification. (White arrow: immunonegative cell, black arrow: immunopositive cell). (Source: Present study).

Caspase-3 expression

Caspase-3 expression significantly decreased with increasing doses of Bevacizumab ($p < 0.05$). The Control group exhibited the highest expression, followed by G1, G3, and G2 in descending order. Statistically significant differences were observed between the Control and G2 groups, as well as between the Control and G3 groups (Figure 4, Table 1).

VEGF expression

An increase in VEGF expression was observed in the G1 group compared to the Control group; however, this increase was not statistically significant ($p > 0.05$). In contrast, significant reductions in VEGF expression were observed in the G2 and G3 groups compared to both the Control and G1 groups ($p < 0.05$). These findings indicate that higher doses of Bevacizumab more effectively suppress VEGF expression (Figure 5, Table 1)

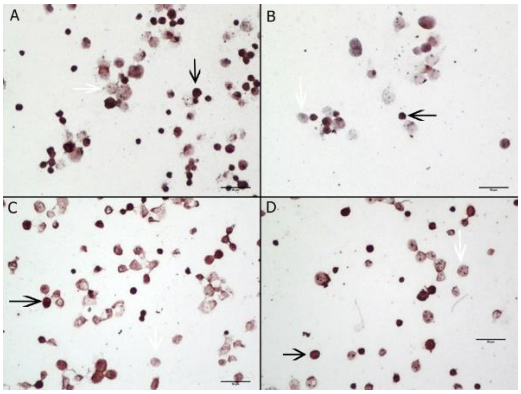

Figure 4. A (Control group), B (Group 1), C (Group 2), D (Group 3) Caspase-3 protein expression in immunopositive and immunonegative cells at ×20 magnification. (White arrow: immunonegative cell, black arrow: immunopositive cell). (Source: Present study).

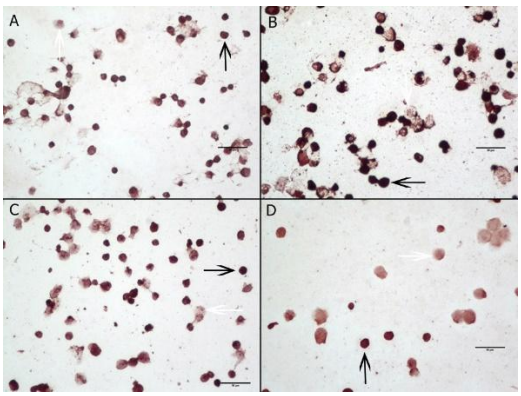

Figure 5. A (Control group), B (Group 1), C (Group 2), D (Group 3) VEGF protein expression in immunopositive and immunonegative cells at ×20 magnification. (White arrow: immunonegative cell, black arrow: immunopositive cell). (Source: Present study).

Statistical analysis results

Immunohistochemically stained cells were counted in each group using light microscopy. For each marker, the ratio of immunopositive to immunonegative cells was calculated (Figure 6).Significant differences were observed between the groups in terms of Bax, Caspase-3, and VEGF expression ($p < 0.05$) (Table 1).

Table 1. Comparison of immunoreactivity levels of Bax, Bcl-2, Caspase-3, and VEGF proteins in groups treated with different doses of Bevacizumab (Control, Group 1, Group 2 and Group 3).

	Bax	Bcl-2	Caspase-3	VEGF
Control	8.63±2.57 [c] (8.34)	0.50±0.26[a] (0.53)	5.10±3.82 [c] (3.70)	2.57±0.72 [bc] (2.65)
Group 1	5.91±2.85 [bc] (6.24)	0.83±0.63[a] (0.77)	1.40±0.49 [bc] (1.23)	3.22±1.02 [c] (2.91)
Group 2	2.05±1.04 [a] (1.93)	0.18±0.13[a] (0.18)	0.49±0.08 [a] (0.48)	0.48±0.13 [a] (0.47)
Group 3	2.70±1.22 [ab] (2.51)	0.18±0.19[a] (0.09)	0.83±0.12 [ab] (0.86)	1.29±0.20 [ab] (1.27)
P*	0.015	0.063	0.003	0.005

Data are presented as mean ± standard deviation, with median values in parentheses. (*$p < 0.05$). Bax protein: Group 2 showed a significant decrease compared to Control and Group 1. Bcl-2 protein: No statistically significant differences were observed between groups ($p = 0.063$).Caspase-3 protein: Group 2 showed a significant decrease compared to Control and Group 1; Group 3 also differed significantly from Control. VEGF protein: Group 2 showed a significant decrease compared to all other groups. Pairwise group comparisons were performed using Dunn's test with Bonferroni correction. The letters indicate which groups show statistically significant differences:
[a]: Indicates a significant difference compared to Group 2.; [b]: Indicates a significant difference compared to Group 3; [c]: Indicates a significant difference compared to the Control group. (Source: Present study)

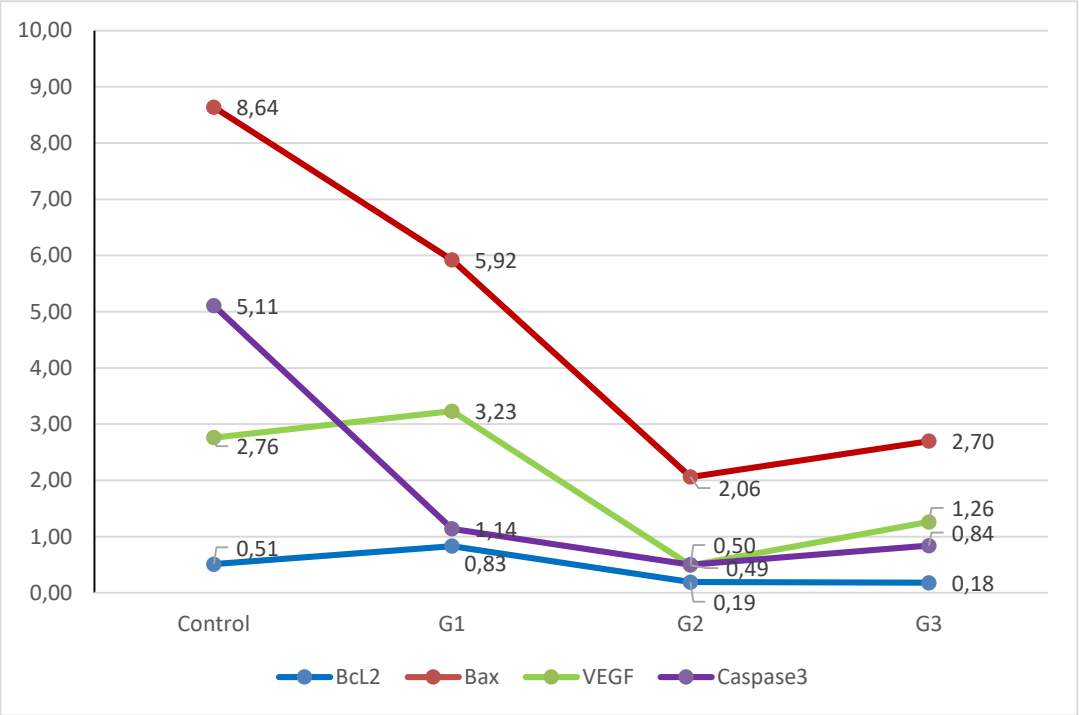

Figure 6. The graph shows the immunoreactivity levels of Bax, Bcl-2, VEGF, and Caspase-3 proteins in the Bevacizumab-treated groups (G1, G2, G3) and the control group.

The graph illustrates the dose-dependent effects of Bevacizumab on the expression levels of these proteins. (Source: Present study).

DISCUSSION

Testicular tumors in young and middle-aged men are generally highly treatable. Their incidence increases with age, and they typically originate from germinal tissue. Despite significant advancements in treatment strategies, serious complications associated with antitumor agents, chemotherapeutics, and, when indicated, radiotherapy continue to pose major clinical challenges. Currently, the most effective treatment for GCTT is the BEP combination. However, this regimen can cause serious side effects, including hearing loss and anemia. Therefore, alternative chemotherapeutic agents are being actively investigated[2,3].

In parallel, we aimed to evaluate the effectiveness of the VEGF inhibitor Bevacizumab in this tumor, which has been the focus of extensive research in recent years. This hypothesis is based on the established role of VEGF inhibitors as monoclonal antibodies that block angiogenesis. Given that invasion and metastasis exacerbate the clinical course of tumors, the effectiveness of these agents has been demonstrated. However, previous studies primarily investigated solid tumor tissues from patients, focusing on the drugs' effects on VEGF activity and angiogenesis.

In our study, we aimed to determine whether Bevacizumab has an antitumoral effect as a VEGF inhibitor. For this purpose, we preferred to conduct a cell culture experiment. Given the rarity of GCTT cases and associated ethical constraints, an established cell line was deemed the most suitable model for this study. Therefore, the study was planned to be conducted with a GCTT cell line obtained from the DSMZ company. However, in our extensive literature search, we found only one study that used the GCTT cell line and the BEP combination[11].

When the study was conducted at this dosage, we observed that even at the lowest dose, cell viability was only 20%, highlighting the need for dosage adjustments. To achieve approximately 50% cytotoxicity, standard dose titration techniques using multiple Bevacizumab concentrations were

employed. Cell viability was measured using the base dose alongside varying Bevacizumab concentrations. Subsequently, the expression of VEGF, Caspase-3, Bax, and Bcl-2 was evaluated immunohistochemically in the same cells. Considering these elements, our approach incorporated parameters not previously assessed even in GCTT tissue studies, providing novel and significant findings. The BEP combination has been used for many years in germ cell tumors[12]. However, recent studies have aimed to increase effectiveness and reduce side effects by adjusting the doses and alternative forms of each chemotherapeutic agent in the BEP regimen. In one study, carboplatin was used instead of cisplatin, but no treatment effectiveness was observed. Moreover, the pegfilgrastim form of filgrastim, which delays cytotoxic chemotherapy, has been shown to significantly reduce side effects during BEP treatment. One serious complication observed during treatment is pneumonia caused by Bleomycin, a component of BEP. In one study, it was shown that removing Bleomycin during this complication did not affect survival[13,14,15].

The Bax gene was the first identified pro-apoptotic member of the Bcl-2 protein family. In cells, most Bax is located in the cytoplasm; however, upon initiation of an apoptotic signal, Bax undergoes a conformational change. It plays a key role in apoptosis induction via the mitochondrial pathway. Bax expression is upregulated by the tumor suppressor protein p53. Bax is essential for p53-mediated apoptosis and functions as a pro-apoptotic factor. The p53 protein, when activated in response to cellular stress, acts as a transcription factor regulating numerous target genes, including Bax[16,17,18].

In our study, the increasing decrease in cell viability with increasing doses of Bevacizumab applied to the cell line demonstrates the cytotoxic effect of Bevacizumab on GCTT cells. However, when looking at the study results, the significant decrease in Bax expression with increasing doses indicates that Bevacizumab does not induce cell death through the Bax pathway in this process.

The Bcl-2 gene, which is encoded in humans, regulates cell death by exerting either pro-apoptotic or anti-apoptotic effects. Bcl-2 is located on the outer mitochondrial membrane, where it supports cell survival and inhibits the activity of pro-apoptotic proteins. Pro-apoptotic members of the Bcl-2 family, including Bax and Bak, normally act on the mitochondrial membrane to rugulate permeability and the release of critical signals, such as cytochrome C and reactive oxygen species (ROS), during apoptosis cascade[19].

In our study, the lack of significant changes in Bcl-2 expression between groups indicates that Bevacizumab does not have cytotoxic activity in this cell tumor type.

Initially known as vascular permeability factor (VPF), VEGF is a signaling protein produced by cells that stimulates blood vessel formation. The VEGF family in mammals comprises five members: VEGF-A, placental growth factor (PGF), VEGF-B, VEGF-C and VEGF-D. although VEGF-A affects multiple cell types, its activity has been primarily studied in vascular endothelialcells. In vitro, VEGF-A stimulates endothelial cell mitogenesis and migration. VEGF-A also functions as a vasodilator, increasing microvascular permeability, which led to its original designation as vascular permeability factor [20,21].

In our study, although there was an increase in the lowest dose group, it was not statistically significant. However, in the G2 and G3 groups, VEGF expression significantly decreased, indicating that Bevacizumab does not have an effect on this tumor cell line at high doses. However, the increase in the G1 group at low dose was disregarded in the evaluation because it was not significant.

Based on these results, we conclude that Bevacizumab exhibits a more pronounced VEGF inhibitory effect at lower doses in the GCTT tumor line, indicating that further studies for dose optimization would be beneficial. However, under in vitro conditions, the VEGF inhibitory effect of Bevacizumab appers limited in this tumor line. Caspase-3 is a member of the cysteine-aspartic acid protease (caspase) family, and the sequential activation of caspases plays a central role in the execution phase of apoptosis. Caspases exist as inactive proenzymes that undergo proteolytic processing at conserved aspartic residues to produce large and small subunits that dimerize to form the active enzyme. These caspases cleave and activate Caspases-6 and -7 and Caspase-3 itself is processedand activated by Caspase-8-9 and -10[22,23]. Caspase-3 is activated in apoptotic cells via both extrinsic (death ligand) and intrinsic (mitochondrial) pathways. The zymogen form of Caspase-3 is necessary, as unregulated, caspase activity would randomly induce cell death[24,25].

The zymogen of Caspase-3 exhibits minimal activity until it is cleaved by an initiator caspase following apoptotic signaling events. Such signaling involves the delivery of granzyme B by NK cells to target cells, which activates initiator Caspases and subsequently triggers the characteristic caspase cascade, in which Caspase-3 playscentral role[26,27].

In our study, although Bevacizumab reduced cell viability in the GCTT cell line in a dose-dependent manner, this effect did not occur via the expected apoptotic pathways. Specifically, the marked decrease in pro-apoptotic Bax expression with increasing doses, together with the unchanged levels of anti-apoptotic Bcl-2 suggest that Bevacizumab does not trigger mitochondrial (intrinsic) apoptosis in these cells. Furthermore, the observed reduction in Caspase-3 expression with increasing doses further indicates that Bevacizumab does not promote cell death though this key apoptotic effector.

These findings suggest that the antitumor effect of Bevacizumab may be primarily attributable to VEGF inhibition rather than involvement in intracellular apoptotic signaling pathways. The absence of apoptotic activity in GCTT cells implies that Bevacizumab mainly acts at the vascular level without directly affecting tumor cell signaling. Moreover, the dose ranges used in this study may have suppressed apoptotic pathways or induced alternative cellular adaptations.

The phase 2 randomized CheckMate 9X8 clinical trial demonstrated that, when combined with Nivolumab, even low-dose Bevacizumab maintained or enhanced clinical efficacy in specific molecular subgroups of metastatic colorectal cancer, including (e.g., microsatellite stable (MSS), proficient mismatch repair (pMMR), consensus molecular subtype 1 (CMS1) and concensus molecular subtype 3 (CMS3)). These findings support a synergistic interaction in which Bevacizumab regulates tumor vascularization while Nivolumab enhances immune activation. This synergy may improve treatment tolerability and reduce toxicity, particularly at lower Bevacizumab doses[9].

The findings of this study demonstrate that low-dose Bevacizumab induces limited apoptotic responses, providing insight into the biological effects of this agent. These data offer preliminary evidence that may serve as a scientific basis for developing strategies to optimize dosing in combination therapy regimens involving immunotherapeutic agents. Therefore, further studies using lower doses and more sensitive detection methods are warranted to better characterize the drug's efficacy profile.

In our study, assays for cell migration, invasion, and colony formation could have been performed, and oxidative stress could have been assessed. Additionally, chemotherapeutic drugs could have been encapsulated in polymers to allow comparison of experimental efficacy. Due to the limited number of studies on this topic and the inability to perform comparative analyses, these factors represent the main limitations of our study.

The results of our in vitro study may be compared with future in vivo animal experiments. Such comparisons could provide important preclinical data, thereby contributing to the literature and informing subsequent clinical studies.

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