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In Vitro Effects of Boric Acid and Bevacizumab in Non-Small Cell Lung Cancer

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Abstract: Lung cancer is one of the most common types of cancer worldwide and is responsible for the loss of more than 1 million people each year. It has been reported that the 5-year survival rate of lung cancer is approximately 15 % or less due to cell metastasis. Therefore, there is a need to develop adjuvant therapies to prevent death from lung cancer cell metastasis. The aim of this study is to evaluate the effects of boric acid and bevacizumab on the vascularization, apoptotic, and metastasis steps of A549 lung cancer cells, such as invasion, migration, and epithelial mesenchymal transition (EMT) abilities, either alone or in combination. The study was divided into 4 groups as control(CONT) and boric acid (BA), Boric acid+altuzan (BA+ALT) and altuzan (ALT). The IC50 dose of boric acid was determined by the MTT method. 30µM boric acid and 7 µM Altuzan were applied to BA, BA+ALT and ALT groups for 24 hours. Anti-VEGF for vascularization, Anti-Vimentin for EMT, Anti-MMP-9 for invasion, and Anti-Bax, Anti-Bcl-2 and Anti-Caspase-3 antibodies for apoptosis were stained immunocytochemically and H-Score analysis was performed. Cell migration was evaluated by the wound healing assay. It was observed that MMP-9 immunoreactivity and apoptotic markers increased in the direction of Cas-3 in the BA group, while the immunoreactivity of Vim and VEGF did not change significantly ($p<0,05$). When the migration was evaluated, it was observed that the cells did not migrate in the BA and BA+ALT groups at the end of the 24th hour, and the wound areas were closed in the other groups. It was observed that while BA affected the migration, invasion and apoptotic characters of A549 cells independently of bevacizumab, it had no effect on their vascularization properties
Keywords: A549; Apoptosis; Bevacizumab; Boric Acid; EMT.

1. Introduction

Because lung cancer is an aggressive type of carcinoma, it is in the first place of cancer-related deaths (1, 2). An estimated 1.1 million deaths due to lung cancer occur worldwide each year (3). Although different treatment options such as antiangiogenic, immunotherapy and radiation therapy are applied for lung cancer, survival rates are quite low. Because of metastases, the survival rate of lung cancer remains

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below 15 percent even for a period of 5 years (4, 5). If a metastasis-preventing treatment can be developed, it can be predicted that survival rates will increase. A recent study showed that transition epithelial to mesenchymal (EMT) plays a key role in cancer metastasis process (6). EMT is expressed by abnormalities in the regulation of EMT markers. Loss of the epithelial marker E-cadherin and increases in the mesenchymal markers vimentin and N-cadherin have been associated with metastatic and invasive behavior. The metastatic behavior of cancer cells was evaluated using these markers in studies. For example, propofol has been shown to significantly increase the E-cadherin expression, while decreasing the expression of vimentin, N-cadherin, and SNAIL, which is considered as evidence that propofol can inhibit the process of EMT (7).

Bevacizumab (altuzan), one of the molecularly targeted, new generation antineoplastic drugs; Its mechanism of action is to inhibit the biological activity of vascular endothelial growth factor (VEGF), and it's shown that it can be used in the treatment of cancers by suppressing the formation of tumor vessels (8). It is frequently used in clinic for the treatment of primary, intermediate and advanced tumors. In addition, recent findings in studies have concluded that bevacizumab may regulate its anti-tumor effect by activating signal cascades of ER's at the cellular level, apart from its ability to inhibit vascular formation (9). It was shown that bevacizumab treatment produced an antitumor effect at the cellular level in A549 cell xenografts (10, 11). It is reported that tumor progression, that is, growth, infiltration and metastasis steps, is supported by tumor neovascularization. VEGF expression has been found to be excessive in other types of cancer, including non-small cell lung cancer (NSLC) and has also been correctly associated with recurrence and metastasis rates (12-14). Bevacizumab is a monoclonal antibody (MAb) which binds specifically to VEGF and can block neovascularization (15). However, Kim et al. suggested that bevacizumab inhibits the apoptosis pathway through activation of Bcl-2 gene expression (16) .

Boron is a trace element for organisms and its derivative in the form of water-soluble inorganic acid, boric acid, is abundant in natüre (17, 18). In a study, boric acid shown to inhibit proliferation while the cell is still in the cell cycle stage, without causing cell death (19). The cytotoxic abilities of boric acid for cancer have been presented by some studies, but its cancer-related cellular effects have not been fully elucidated (20, 21). Knowing the cellular changes caused by boric acid will reveal its potential therapeutic effects and will guide other anticancer studies in this field (22).

We aimed to evaluate the possible effects of single and combined applications of boric acid and bevacizumab on A549 lung cancer cells in a two-dimensional cell culture environment using apoptotic and EMT transition markers.

2. Material and Methods

2.1. Preparation of Chemicals

Boric acid was used by dissolving 30 µM in a medium heated to 37 °C and passing through a 0.22 µm filter. Altuzan (100 mg/4ml, Roche) was used by dissolving 7µM in a medium heated to 37 °C.

2.2. Cell Viability Determination by MTT

MTT test was performed for determinening the cytotoxic effect of boric acid on cells A549 and IC50 dose. A549 non-small lung cancer cells used $2x10^4$ cells per each well, and 10, 30, 50 and 100 µM boric acid was applied 24 hours. After 24 hours, MTT (Sigma-Aldrich) performed as desciribed bey manifacturer before. Values were measured in a microplate reader (Shimadzu UV-1601). And the MTT assay performed for 8 times. For Altuzan, on the other hand, since there are sufficient number of dose determination studies in the literature, no dose determination was made and 7 µM was used in accordance with the literature (23).

2.3. Culture of Cells

A549 non-small lung cancer cell line (n=4) was incubated with DMEM F12 (SLM-243-B, Sigma) medium containing 10 % Fetal bovine serum (FBS)(10270106, Gibco) 1 % L-Glutamine (59202C, Sigma) and 1 % antibiotic- (penicillin-streptomycin) (P4333, Sigma) in a 37 ˚C humid incubator with 5% carbon dioxide. When 80 % confluency was reached, cells were divided into four treatment groups. The groups were grouped as Boric acid (BA), Altuzan (ALT), boric acid+Altuzan (BA+ALT), and control (CONT) groups, IC50 doses of the chemicals determined by MTT were applied to the cells for 24 hours.

2.4. Immunocytochemical Staining

The determined groups were were fixed with paraformaldehyde 4 % (PFA) 30 min. After fixation, hydrogen peroxide 3 % (H_2O_2) 5 min was applied. For permeabilization, was performed with Triton-X 0.1 % 100 and proteins were blocked for an hour. Anti-Vimentin (1/200, ab8978, abcam), anti-VEGF (1/200, ab76055, abcam), anti-MMP-9 (1/200, ab76002, abcam), anti- Bcl-2(1/200, sc-7382-santa cruz), anti-Bax (1/200, MA5-14003-thermo), and anti-Caspase 3 (1/200, sc-56053) incubated 1 night at +4 ºC. Secondary antibodies applied and visualized with DAB, photographed with a (NIKON Eclipse E600) light microscope. H-Score evaluation and statistical analysis were performed. H-score: 500 cells from each field were counted per groups. The staining intensities; The number of stained cells (%) evaluated using this formula X (Staining intensity 0, 1, 2, 3, 4, 5), with a H-score between 0 and 500 (24).

2.5. Assay of Cell Migration

Wound healing assay was applied to evaluate the migration tendencies of cells. A549 non-small lung cancer cells from ATCC (CCL-185) were incubated until 100 % confluent. Afterwards, the groups were applied at the determined drug doses, and a wound area was created between the cells by drawing a line from the middle area with the a sterile 10 µl pipette tip. In order to compare the tendency of the cells to migrate towards the to area and close the wound area and their speed during this migration, photographs were taken from the groups at 12 hour intervals with a phase contrast microscope (ZEISS Axio Vert iLED) and comparatively evaluated (25).

2.6. Statistical analysis

The results of the H-score evaluation and the measurements taken in the cell migration experiment were analyzed statistically using the SPSS 20.0 (demo version) statistical analysis program, using Kruskal Wallis test for comparison of groups and Dunn test for pairwise comparisons.

3. Results

3.1. Evaluation of the Effect of Boric Acid on Cell Viability by MTT Method

Cell viability was determined by MTT method to determine the IC50 dose of boric acid in A549 cells. A549 cells were treated with 10, 30, 50 and 100 µM boric acid for 24 hours. After 24 hours, it was seen that 100 µM dose killed all cells. In the cell groups treated with 10, 30 and 50 µM, the average viability rates were observed as 81.54 % and 27 %, respectively. The IC50 dose of BA for A549 cells was determined as 30 µM and applied to the study groups in other steps (Figure 1).

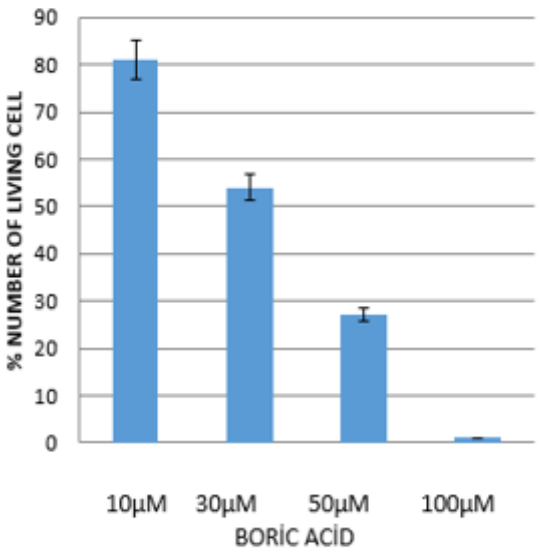

Figure 1. MTT analyzes of the effects of boric acid tretment on cell viability in A549 cells.

3.2. Effects of Boric Acid and Altuzan on Epithelial Mesenchymal Transition

In order to immunocytochemically evaluate the effect of BA and Boric acid+Altuzan on epithelial mesenchymal transition, boric acid, boric acid+Altuzan and altuzan were applied to A549 cells for 24 hours. The cell group that did not receive any treatment was accepted as the control. After the treatments, Vimentin protein expression was evaluated by immunocytochemical method and by H-Score analysis (Table 1). According to the H-Score analysis results, the immunoreactivity of Vimentin did not differ significantly in the other groups compared to the control group ($p>0.05$).

3.3. Effects of Boric Acid and Altuzan on Invasion

The effect of boric acid and Altuzan on cell invasion was evaluated immunohistochemically by the expression of MMP-9 protein. H-Score analyzes were performed on the differences in expression intensity of MMP-9 protein between the groups (Figure 2). There were a significant decrease in MMP-9 immunoreactivity in the BA and ALT groups (respectively $p=0.042$, $p=0.002$) and in the BA+ALT group compared to the control group ($p=0.004$) (Table 1).

3.4. Effects of Boric Acid and Altuzan on Cell Migration

A549 cells were incubated in 24-well culture dishes to allow proliferation of cells to cover 80% surface area. After providing full coverage of the cells on the surface, the wound healing test, which is often used to evaluate the migration ability, was performed. For BA, BA+ALT and ALT groups, doses determined by MTT were applied. The cell group that did not receive drug treatment was accepted as the control. The migration of cells to the wound area was monitored for 24 hours, and the wound areas were measured and photographed for each group at 0, 12, and 24 hours. At the end of the 24th hour, complete closure was observed in the groups and the experiment was terminated and the areas in the groups with gaps were measured in µm² and compared. The experiment was repeated 4 times for each group and statistical analyzes were made with pairwise comparison tests. Complete closure was observed in the control group and ALT group at the 12th hour. When BA and other groups were compared at the 24th hour, no significant difference was observed between the BA+ALT group and BA, but there was a significant difference between CONT and BA ($p= 0.002$), and ALT and BA ($P=0.017$). When statistical analyzes are evaluated, it is seen that this significant difference is due to BA (Figure 3, Table 2).

3.5. The Effects of Boric Acid and Altuzan on Apoptosis

In order to evaluate the effect of Boric acid and Altuzan on cell apoptosis in A549 cells, the expression of Bax, Bcl-2 and Cas-3 proteins was determined by immunocytochemical method and analyzed by scoring

with H-Score method after 24 hours of treatment. According to the results of the H-Score analysis, when Bax immunoreactivity was compared with the CONT group, an increase was observed in the BA and BA+ ALT groups (p=0.002, p= 0.042, respectively). The difference between the CONT group and the ALT group was not significant (p=0.308). When ALT and BA+ALT groups were evaluated, it was observed that there was a decrease in Bcl-2 immunoreactivity compared to the CONT group (p= 0.054). There was no significant difference in the BA group compared to the CONT group (p=0.258).

While Cas-3 immunoreactivity increased significantly in the BA group compared to the CONT group (p=0.024), the increase in the BA+ALT group was not significant (p>0.05).

3.6. The Effects of Boric Acid and Altuzan on Vascularization

In order to evaluate the effect of boric acid and Altuzan on vascularization in A549 cells, after 24 hours of treatment, the change in VEGF protein expression was evaluated by immunocytochemical method and H-Score analysis was performed (Figure 2). According to the results of H-Score analysis, there was a significant decrease in VEGF immunoreactivity in the ALT group (p=0.003) and BA+ALT (p= 0.029) groups when compared to the CONT group, and these differences were significant.

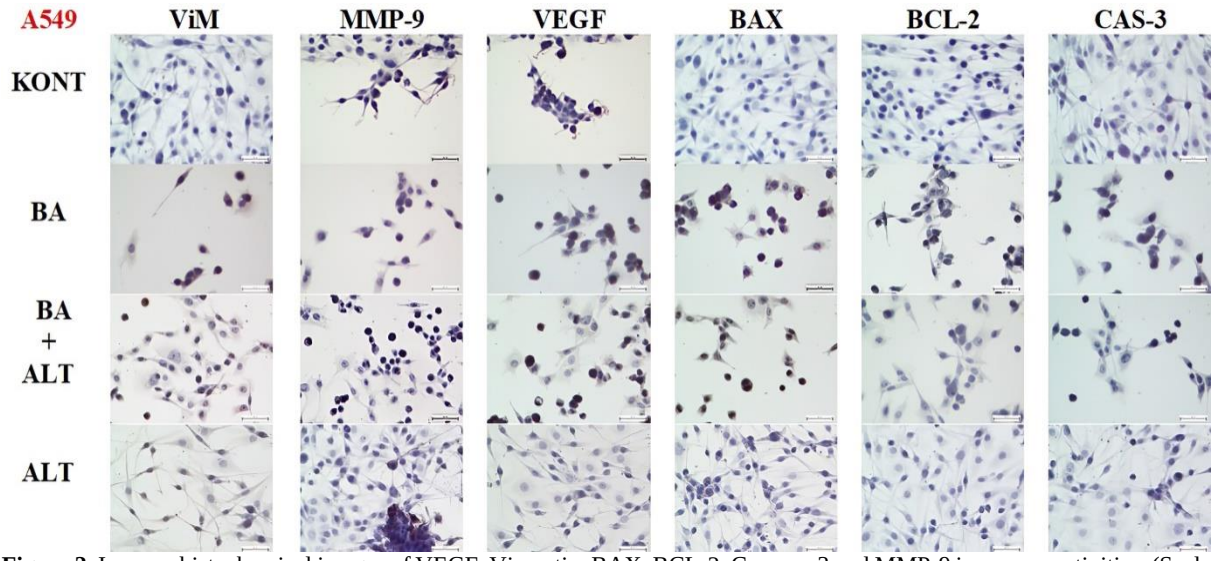

Figure 2. Immunohistochemical images of VEGF, Vimentin, BAX, BCL-2, Caspase-3 and MMP-9 immunoreactivities. (Scale Bar 100 µm).

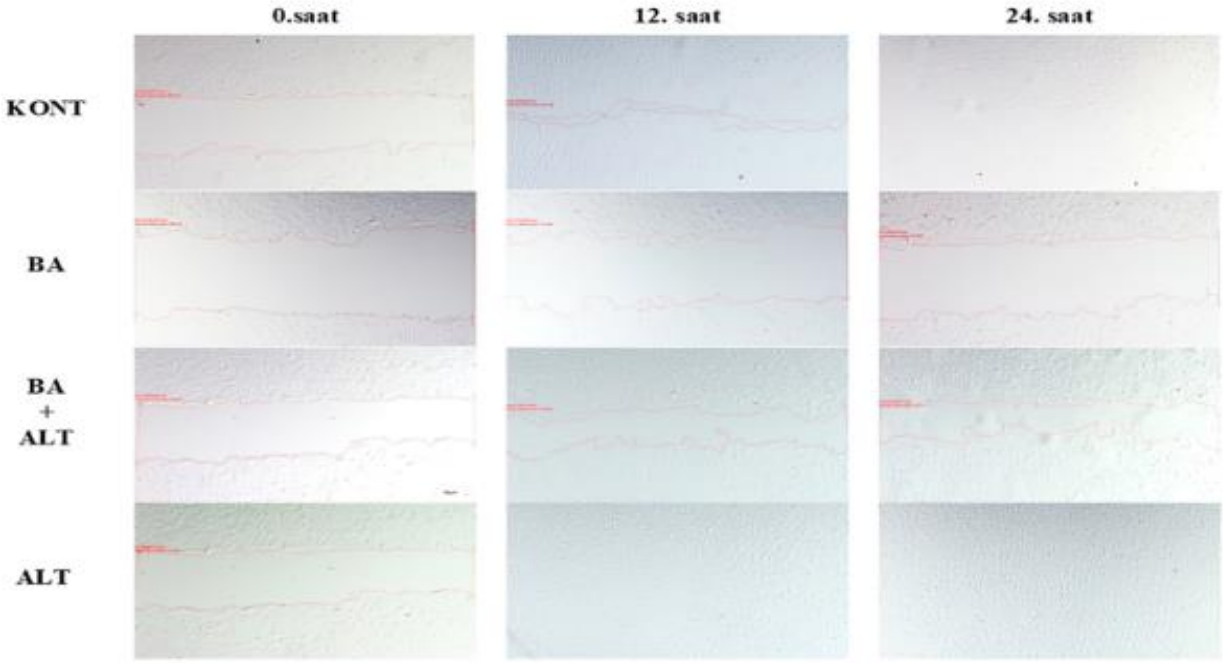

Figure 3. Evaluation of cell migration by wound healing assay (Scale bar 200 µm).

Table 1: H-Score pairwise comparison statistical differences between groups.

Groups	Vimentin	MMP-9	VEGF	Bax	Bcl-2	Cas-3
BA	255±3.60^{ab} (255)	186±1.90^{ab} (186)	234±5.13^{a} (236)	263±2.45^{bc} (264)	185±4.84 (185)	242±2.63^{ab} (243)
ALT	223±3.78^{a} (224)	202±4.16^{c} (200)	152±2.55^{ab} (152)	207±2.91^{c} (208)	180±2.33^{b} (180)	185±2.11^{a} (185)
BA+ALT	226±1.50^{b} (227)	234±3.61^{ab} (225)	183±2.55^{c} (184)	183±4.27^{a} (226)	177±1.80^{a} (177)	212±3.00 (212)
CONT	250±2.15 (250)	272±3.00^{abc} (272)	242±4.10^{bc} (242)	150±4.16^{ab} (150)	232±3.00^{ab} (232)	190±6.00^{b} (190)

Different letters in the line indicate significant difference between groups.

Table 2: Pairwise comparison statistical differences between cell migration experimental groups.

Groups	0. HOUR	12. HOUR	24. HOUR	P Value
CONT	51798.35±7559.54$^{\mathbf{aA}}$	1356.33±498.26$^{\mathbf{aA}}$		0.018
BA	70066.61±14729.28$^{\mathbf{aA}}$	77961.70±5319.95$^{\mathbf{aB}}$	70717.83±8413.03$^{\mathbf{aA}}$	0.174
ALT	55913.25±8994.52$^{\mathbf{bA}}$	1425.62±299.9^{abA}	1166.75±119.33$^{\mathbf{aB}}$	0.039
BA+ALT	55746.54±8981.36$^{\mathbf{bA}}$	35138.58±4387.13$^{\mathbf{abAB}}$	30247.35±8353.02$^{\mathbf{aAB}}$	0.039
P Value	0.126	0.005	0002	

Different lowercase letters in the row and different uppercase letters in the column indicate significant differences between groups.

4. Discussion

Lung cancer is the leading cause of cancer-related deaths worldwide, with approximately 2 million new cases and 1.76 million deaths annually. The treatment of cancer may include multiple options, such as radiation therapy, surgery, chemotherapy, hormone therapy, alternative medicine, or targeted therapy, depending on the patient's overall health. Many anti-cancer substances used in alternative medicine have been identified, produced and tested to date, and 70% of the anti-cancer drugs used are obtained from natural sources (26). Boron, after silisyum, is one of the most common elements in natüre (27). It is usually found in nature as a chemical compound such as boric acid (BA), and BA is the most common form of boron found in the human body (28). Many researchers have pointed out that boric acid has antioxidant, anticancer, antigenotoxic and hepatoprotective properties (29, 30). In the present study, the IC50 dose of boric acid application, which is considered a toxic dose, in A549 lung cancer cells was investigated by the MTT method and determined as 30 μM. The determined dose was used to establish the treatment groups. The possible effects of boric acid on the migration, invasion, apoptosis and EMT properties of non-small cell A549 lung cancer cells, alone and together with Altuzan, which is used in cancer treatment, were investigated by in-vitro methods.

Vimentin is an important component of the intermediate filament family of proteins and is expressed in normal mesenchymal cells. It is known to maintain cellular integrity and provide resistance to stress. Overexpression of vimentin in cancer is well associated with increased tumor growth, invasion, and poor prognosis. In recent years, vimentin has gained importance as a marker for epithelial-mesenchymal transition (EMT) (31). In our study, when compared with the control group, it was observed that there was no significant change in Vimentin immunoreactivity in all groups. It was thought that the use of boric acid alone or in combination with altuzan did not alter vimentin protein expression during 24-hour treatment, so it had no effect on mesenchymal transition through vimentin protein. The limited studies in the literature regarding the effects of boric acid on vimentin protein expression in non-small cell lung cancer cells make it difficult to evaluate epithelial mesenchymal transition in terms of vimentin protein. In addition, the study we presented seems to be a peereview study for other researchers. When the effects of BA on the migration of cells were evaluated with the cell migration-migration experiment, there were significant differences in the BA and BA+ALT groups at the end of 24 hours compared to the other groups and the cells migrated towards the wound area in the ALT and CONT groups and completely covered the area, the migration characteristics of the cells were determined by BA shows that it is affected. The absence of changes in vimentin proteins, which are used as markers for EMT transition, suggests that the invasion abilities of cells are suppressed and the EMT transition may have never been initiated.

Cancer cells secrete serine proteinases and matrix metalla proteinases (MMPs) that break down extracellular matrix proteins in order to migrate into the tissue. Highly expressed MMP-2 and MMP-9 are in malignant tumor cells, facilitating cell migration by playing a role in the degradation of the extracellular matrix, which is an important component of the basement membrane that leads to cancer metastasis (32). MMPs are genes involved in cancer disease functional promotion of angiogenesis, progression, metastasis, invasion, and escape from control of immune cells, these genes have been noted to be frequently upregulated in cancer (33). In our study,

MMP-9 immunoreactivities were also found to be significantly decreased in the BA, ALT and BA+ALT groups compared to the CONT group. Jiang et al. In this study, the effect of local treatment of bevacizumab, the active ingredient of altuzan, against triple negative breast cancer (TNBC) xenograft tumors was examined and they said that MMP-9 expression level decreased compared to the control groups (34). In our study, a significant decrease in MMP 9 immunoreactivity in the BA group compared to the CONT group suggests that it has a similar effect independent of the altuzan effect. Contrary to our results, Wang D. et al. in 2015 showed that bevacizumab strongly inhibited MMP 2 and MMP 9 in A549 cells in a dose-dependent manner at the mRNA level (35). The difference in our results is that the effective dose of bevacizumab used in this study showed a similar effect at twice the values we used. When we look at the literature studies, it seems that the number of studies showing and discussing the direct effects of boric acid on MMPs is quite limited. In a study conducted with boron, which is also a derivative of boric acid, the effects of boron on keratinocytes MMP 2 and MMP 9 were examined for the evaluation of wound healing in vitro, but contrary to our study, it was observed that it accelerated the wound healing process by increasing the amount of MMP (36). This difference was thought to be due to the experiment with non-cancerous keratinocytes.

Bevacizumab is the active ingredient of Altuzan, an anti-vascular endothelial growth factor (VEGF) monoclonal antibody (mAb) and a drug used in various types of cancer (37). Vascular endothelial growth factor (VEGF) is an endothelial cell-specific mitogen and an angiogenic inducer in various in vivo models. VEGF is believed to be one of the key molecules as it stimulates endothelial cell activation, survival and proliferation of tumor cells and facilitates invasion and migration (38). VEGF overexpression has been observed as a poor prognostic parameter in lung carcinomas (39). In our study, when VEGF immunoreactivities were compared with the control group, the decrease in the ALT group was found to be statistically significant ($p=0.003$). This result was similar to the results of previous studies and showed that altuzan also suppressed angiogenesis in A549 cancer cells. Compared to the control group, the decrease determined when the BA group was evaluated was not statistically significant ($p>0.05$), while there was a significant decrease in the BA+ALT group ($p=0.045$). The decrease in ALT and BA+ALT groups was thought to be due to altuzan. The studies of Özyarim and Karabağ Çoban support our results and show that the application of boric acid at different doses and hours in colon cancer cells did not cause a significant change in VEGF amounts compared to the control group. Karaca et al. in their in vivo study in 2022, they said that boric acid reduces VEGF expression, which is inconsistent with the results of our study. This difference is probably due to the fact that the study was performed in vivo and in non-cancerous tissues or due to the difference in the amounts of boric acid used.

There are studies supporting the use of boric acid in cancer treatment. In some epidemiological studies, BA exposure has been shown to reduce prostate cancer cell proliferation in men and cervical cancer cell proliferation in women, leading to a reduction in the incidence of lung cancer, as well as induce apoptosis in prostate, melanoma, and breast cancer cell lines (40–44). Apoptosis is one of the most accepted cellular death mechanisms for anticancer activity. Although it is believed that only a limited part of the mechanism of apoptosis has been elucidated, activation of caspase family proteins is shown as initiator and maintainer of apoptosis (45,46).It is known that caspase-3 plays the key role in the apoptotic process and that caspase-3, which causes DNAse activation, has a direct effect on DNA fragmentation (47,48). In a study, Boron Nanoparticle spheres were shown to induce apoptosis and inhibit cell proliferation for both androgen-sensitive LNCap and androgen-independent DU145 prostate cancer cells, and also increased caspase-3/7 activity (49). In the present study, changes in the immunoreactivity of the caspase regulatory proteins Bax, Bcl-2 and caspase-3 were evaluated for the evaluation of apoptosis. It was observed that Bax increased significantly in the BA and BA+ALT groups compared to the control, and simultaneously, Bcl-2 immunoreactivity was significantly decreased in the same groups. The increase in Cas-3 immunoreactivity also suggests that the apoptotic process of cells in these groups may be initiated by boric acid and may be regulated by disturbing the balance between Bax/Bcl-2 proteins. It is seen that there is a significant increase in Bcl-2 protein amounts in the ALT group compared to the control. This increase is thought to be due to the effect of bevacizumab, and in support of this idea, there are studies in the literature showing that bevacizumab can have a bidirectional effect at certain dose ranges (23).

In our study, we investigated the possible effects of Boric Acid and bevacizumab on the migration, invasion, apoptosis and EMT properties of non-small cell lung cancer cells A549 in-vitro. According to the findings and results of the methods we used, BA affects the migration, invasion and apoptotic characters of A549 cells independently of bevacizumab. In our study, it was not observed that there was any effect on VEGF-mediated vascularization. Although our study is among the pioneering studies in which the possibility of using BA for therapeutic purposes is questioned, it is a study that was conducted in an in vitro 2D environment and has limitations. On the other hand, we think that it will shed light on the literature as a pre-study.

5. Conclusions

Our study presents the effect of boric acid on the alteration of protein expressions, which are effective in the EMT process of lung cancer cells. Further 3D studies, animal and human experiments are needed to elucidate the therapeutic effects of boric acid on EMT.

Limitations of the Study

The limitations of this study are that it is a cell culture study arranged in a two-dimensional environment.

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Conflict of Interests

The authors declared no conflict of interest.

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Author Contributions

ECT and ZO designed the research. ECT participated in data collection and data analysis. ECT and ZO wrote the manuscript, read and approved the final script

Ethical Approval

No Ethics Committee Application İs Required.

Informed Consent

The authors accept their responsibilities in the study. There is no conflict of interest between the authors.

Availability of Data and Materials

The material used in the study and without the permission of the authors.

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