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INVESTIGATION OF APOPTOTIC AND ANGIOGENİC EFFECTS OF BORON IN HUMAN LUNG CANCER CELLS (A549)

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ABSTRACT

Today, lung cancer, one of the most important health problems, is the most common cause of mortality in both men and women. A549 lung cells derived from human alveolar carcinoma cells match the type II alveolar cell phenotype, have many characteristics of human primary alveolar epithelial cells. Boron is used in many fields such as , nuclear, glass, ceramics, pharmaceuticals, detergents, agriculture and its usage areas are increasing day by day. Recent studies have shown that boron is an important element for human health. Based on this information, this study investigated the effect of different concentrations of boric acid in the A549 human lung cancer cell line, by analyzing proliferation assay, TAS (Total Antioxidant Status), TOS (Total Oxidant Status), VEGF (Vascular Endothelial Growth Factor) and PARP (Poly (ADP-). Ribose Polymerase). Proliferation assay was performed using CCK8 Assay Kit. TAS, TOS, VEGF and PARP analyzes were performed using Sun-Red Human Total Antioxidant Status Elisa Kit, Sun-Red Human Total Oxidant Status Elisa Kit, Sun-Red Human (VEGF) Elisa Kit and Sun-Red Human (PARP) Elisa Kit, respectively. it is made. In the study, the proliferation test showed that the viability values decreased in proportions with doses at 5 mM, 10 mM, 20 mM, 40 mM and 100 mM concentrations and the IC50 value was determined as 20 mM. There was no significant difference between TAS and TOS analysis. In VEGF values, it was observed that VEGF values decreased at 10 mM and 20 mM boric acid concentrations compared to the control group, but there was no significant difference in 40 mM boric acid concentration. A significant increase in PARP values was observed at all concentrations of 10 mM, 20 mM and 40 mM. As a result; We may be think 10 mM, 20 mM and 40 mM concentrations of boric acid may decrease angiogenesis by decreasing VEGF levels. As a result of the increase in PARP values, we may be think boric acid leads to cell necrosis at concentrations of 10 mM, 20 mM and 40 mM.

KEYWORDS: Boron, lung cancer, VEGF, PARP.

1. INTRODUCTİON

Today, lung cancer, one of the most important health problems, is the most common cause of mortality in both men and women.[1-3] More than one million people decease each year out of for the treatment of lung cancer in the world. Since 1985, the number of lung cancer cases has increased by 44% in men and by 76% in women worldwide.[3] While lung cancer has tripled since 1985, other cancer types have shown a decrease in mortality rates.[4] The prognosis of lung cancer is worse than other types of cancer, and the five-year survival rate is less than 15%.[5]

A549 cells were isolated by D.J. Giard et al. in 1972 from the lung carcinoma of a 58-year-old man from Central Asia.[6] These isolated cells were cultured for 1000 generations and it was concluded that these cells were immortal.[7] There are two types of alveolar cells. Type I covers 96% of the inner surface of the alveoli and is difficult to be produced in vitro envorinment. Type II, on the other hand, occupies less surface and has many functions. In addition, type II cells are ancestor cells of type I cells. A549 lung cells originated from human alveolar carcinoma cells match with the type II alveolar cell phenotype, and share many characteristics with human primary alveolar epithelial cells.[8,9] It is therefore widely used not only in lung cancer models, but also in in vitro studies of human alveolar epithelial cells.[10-13,7] Cells proliferate by adhering on a single surface and the doubling time is approximately 22 hours.[14]

Boron, which is used in more than 400 areas from nuclear, glass, ceramics, pharmaceuticals, detergents, agricultural and fertilizer industry to the automobile industry, is an element the usage field of which increase

day by day. Given its current uses, boron is said to be the most strategic mine in the world.[15,16]

Recent studies have shown that boron is an important element for human health.[17-23] Boron, existent especially in the structure of bones and teeth, plays crucial role in the absorbtion of calcium, phosphorus and magnesium. Therefore, it is a vital element for bone health. Indeed, daily boron supplementation has been shown to be effective in the treatment of osteoporosis.[18] In a study, it was reported to be effective coronary heart disease.[19] Based on this information, this study was conducted to investigate the effect of different concentrations of boron on the A549 human lung cancer cell line.

2. MATERİALS AND METHODS

2.1. Materials

Human lung alveolar basal epithelial cells used in the study (A549), was obtained from Assoc. Dr. Ömer Hazman, Afyon Kocatepe University Department of Molecular Biology and Genetics.

2.2. Method

2.2.1. Sterilization Before Cell Culture

All plastic materials used in cell culture and ready-made sterile media were obtained from commercial companies. Following the incubations, the lysate and medium samples prepared from the cells were analyzed using the methods described below.

2.2.2. Cell culture

The main content of the medium used in the culture of A549 cells is RPMI 1640, and it contains 10% fetal bovine serum (FBS) and 1% penicillin / streptomycin. Cell culture was performed by being incubated under sterile conditions in a carbon dioxide incubator providing 5% CO2 and 37oC temperature conditions in sterile prepared medium containing T25 and T75 flasks.

Passaging was performed when the cells being amplified reached a density of approximately 85% of the culture flask. Cells were used in experiments when they reached a sufficient number.

2.2.3. CCK 8 Cell Viability Test

For the CCK 8 cell viability test, the cells were seeded in a 90-well plate at 90 tal in RPMI medium containing 10% FBS and 1% penicillin / streptomycin at 90 vel and incubated in a 5% CO 2 and 37°C incubator. After 24 hours, the wells were divided into the following groups (Table 1). Three repetitions of each group were performed.

Table 1: Working Groups.

Groups	Applications
Group 1	RPMI containing 10% FBS and 1% penicillin / streptomycin
Group 2	30 µM Cis Platin
Group 3	100 mM Boric Acid
Group 4	75 mM Boric Acid
Group 5	40 mM Boric Acid
Group 6	20 mM Boric Acid
Group 7	10 mM Boric Acid
Group 8	5 mM Boric Acid

After the applications, they were allowed to incubate for 48 hours. After 48 hours, 10 CCl of CCK 8 solution was added. Incubation was allowed for 2 hours. At the end of the incubation period, they were shaken for 10 seconds and were measured at 450 nm wavelength with plate reader.

2.2.4. PARP Analysis

Cells in 75 cm² flask were used for PARP analysis. Cells were removed with the aid of trypsin. An than they were centrifuged and supernatat was discarded and the resulting pellet were turned into suspension with the addition of medium.

24-well cell culture plates were used for the experiment. The A549 cell was seeded in wells at a density of $5x10^5$. The plate was allowed to stand in the incubator for 24 hours and at the end of 24 the cells from the incubator were treated with various concentrations of Boron. It was placed in a 37ºC incubator for 48 hours. After 48 hours, the cells were removed from the incubator. Media from the plated cells were removed and treated with approximately 700µl of PBS. PBS was withdrawn from the treated cells. 200µl trypsin was added to the cells and placed in a 37ºC incubator and cells were lifted. 450µl of medium was added to the cells separated from the surface to eliminate the effect of trypsin. The cells were transferred to the ependor for PARP by means of a disposable pipette. The cells in the epondorph were centrifuged at 2100 rpm for 15 minutes at 15ºC. After centrifugation, the supernatant was discarded. The medium was added to the ependorf and gently mixed, then added to a 96-well plate loaded with single-use antibody in the kit. Reagents, samples and standards were prepared. Prepared samples and standards were seeded in a 96-well plate and allowed to incubate at 37°C for 60 minutes. After incubation, the wells were washed with wash solution five times each, Chromogen solution

A and B were added, incubated at 37°C for 10 minutes. The stop solution was then added and the measurement was performed.

2.2.5. VEGF Analysis

Cells in 75 cm² flask were used for PARP analysis. Cells were removed with the aid of trypsin. An than they were centrifuged and supernatat was discarded and the resulting pellet were turned into suspension with the addition of medium.

24-well cell culture plates were used for the experiment. The A549 cell was seeded in wells at a density of $5x10^5$. The plate was allowed to stand in the incubator for 24 hours and at the end of 24 the cells from the incubator were treated with various concentrations of Boron. It was placed in a 37ºC incubator for 48 hours. After 48 hours, the cells were removed from the incubator. Media from the plated cells were removed and treated with approximately 700µl of PBS. PBS was withdrawn from the treated cells. 200µl trypsin was added to the cells and placed in a 37ºC incubator and cells were lifted. 450µl of medium was added to the cells separated from the surface to eliminate the effect of trypsin. The cells were transferred to the epondor for VEGF analysis with a disposable pipette. The cells in the epondorph were centrifuged at 2100 rpm for 15 minutes at 15ºC. After centrifugation, the supernatant was discarded. The medium was added to the epondorf and gently mixed, then added to a 96-well plate loaded with single-use antibody in the kit. Reagents, samples and standards were prepared. Prepared samples and standards were seeded in a 96-well plate and allowed to incubate at 37°C for 60 minutes. After incubation, the wells were washed with wash solution five times each, Chromogen solution A and B were added, incubated at 37°C for 10 minutes. The stop solution was then added and the measurement was performed.

2.2.6. Statistical Analysis

The data were analyzed using SPSS-18 computer program and the results were given as mean ± standard deviation (SD). After the homogeneity of the groups was tested, Duncan were used in one-way ANOVA test to find differences between groups. Differences were considered statistically significant at $P<0.05$.

3. RESULTS

3.1. Proliferation Test Finding

A549 Dose ranges for lung cancer cell line were determined as 5, 10, 20, 40, 75, 100 mM. At the end of 24, 48 and 72 hours, the inhibition 50 (IC50) dose was determined as 20 mM bu using CCK8 kit due to %50 death rate of cancer cells and the viability of cancer cells gave better results at 48 hours (Figure 1). Other experiments were based on this time and concentration.

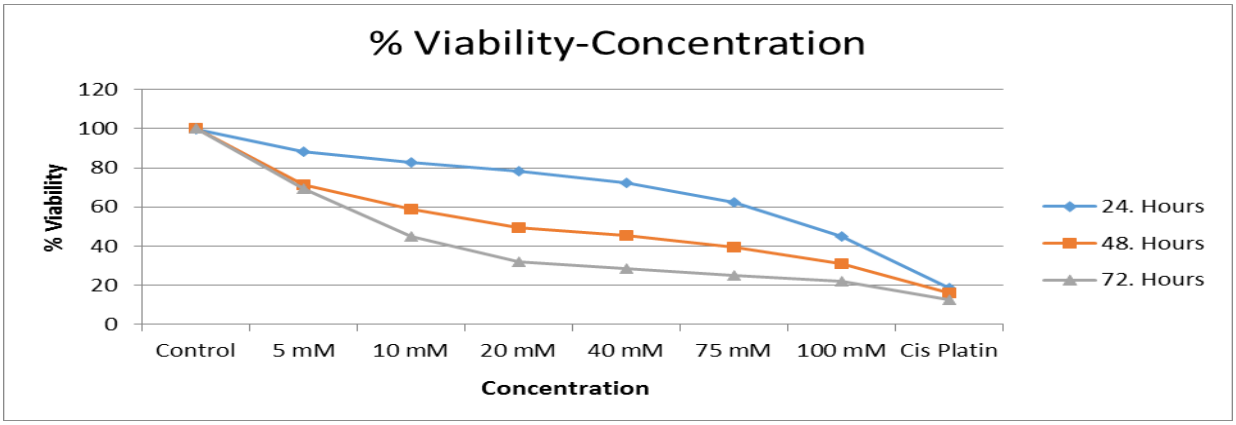

Figure 1: Viability-Concentration Graph.

3.2.PARP and VEGF Analysis Findings

PARP and VEGF analyzes were performed on A549 cell line treated with boric acid at 10 mM, 20 mM and 40 mM concentrations. The results are given in Table 2.

Table 2: PARP and VEGF results.

	PARP (ng\L)	VGEF (ng\L)
Control	0,2480± 0,04[a]	0,63 ± 0,02[a]
Bor 10 mM	0,6881± 0,08[b]	0,074 ± 0,01[b]
Bor 20 mM	0,6219± 0,01[b]	0,068 ± 0,01[b]
Bor 40 mM	0,6560± 0,08[b]	0,062 ± 0,006[b]
Cis Platin	0,6365± 0,03[b]	0,53 ± 0,05[c]

**a,b,c: Means in the same column by the same letter are not significantly different according to the one way ANOVA-Duncan test ($P<0.05$).

3.3. TAS and TOS Analysis Findings

There was no significant change in TAS and TOS analysis.

4. DİSCUSSİON

Boron, which is used in more than 400 areas from nuclear, glass, ceramics, pharmaceuticals, detergents, agricultural and fertilizer industry to the automobile industry, is an element the usage field of which increase

day by day. Given its current uses, boron is said to be the most strategic mine in the world.[15,16]

Recent studies have shown that boron is an important element for human health.[17-23] Boron, existent especially in the structure of bones and teeth, plays crucial role in the absorbtion of calcium, phosphorus and magnesium. Therefore, it is a vital element for bone health. Indeed, daily boron supplementation has been shown to be effective in the treatment of osteoporosis.[18] In a study, it was reported to be effective coronary heart disease.[19] Based on this information, this study was conducted to investigate the effect of different concentrations of boron on the A549 human lung cancer cell line. In a study; in human prostate cancer cells, the cell line DU-145 was used and the presence of boric acid suppressed the growth rate.[24] In another study, the effect of boric acid was analyzed on MDA-MB-231 and MDA-MB-435 estrogen receptor negative human breast cancer cell lines, but the dose of 1mM boric acid did not show any effect on both cells. Positive cell lines MCF-7 and T47-D also showed no significant inhibition. It is envisaged that the reason for this may be different cell mediums that are used in flasks. In the measurements taken on the 7th day, there was a 15% growth inhibition in the estrogen receptor negative SK-BR-3 cell line and 40% growth inhibition in the estrogen receptor positive cell line ZR-75-1. In the light of these studies, it was predicted that the inhibitory effect of boric acid was not responsive to steroid hormone metabolism. Based on these data, the role of boric acid in the mechanism of inhibiting tumor growth has not been understood.[25] Molecular studies have shown that boric acid binds to cis-diols on fragments of nucleotides, including ribose, NAD. From this point of view, researchers have thought that boric acid with nucleotides, nucleotide-borate complexes are formed that by combination of boric acid with nucleotides antiproliferatively affect the functioning or use of nucleotides.[26,27] In another study, Scorei R. et al. investigated the effects of boric acid and calcium fructobate on MDA-MB-231 breast cancer cell line. The effects of different doses of boric acid and fructoborate (0.45-22.5 mM) on cell viability were investigated by 3-(4,5-dimethylthiazolyl-2) -2,5-diphenyltetrazolium bromide (MMT) method. CF and BA have been shown to induce a concentration-dependent cytotoxicity in MDA-MB-231 cells.[28] Ceyhan et al. investigated DU-145 Human Prostate Cancer Cell Line high concentrations of boric acid oxidative stress, apoptotic pathways and morphological changes. The effects of boric acid (0–16,15 mM) applied at varying doses on cell viability after 24 hours were examined by MTT. Boric acid-containing cells have been found to cause a statistically significant decrease in cell viability compared to control group.[29] In this study, 5mM, 10 mM, 20 mM, 40 mM and 100 mM boric acid were applied and a direct proportional decrease in cell proliferation was observed in the A549 lung cancer cell line at 24, 48 and 72 hours. However, after a very high antiproliferative effect in the 72th hour data, other studies were conducted over a 48 hour incubation period. Ceyhan et al. DU-145 examined the effect of high concentrations of boric acid on oxidative stress in the human prostate cancer cell line. TAS-TOS parameters were used to measure oxidant and antioxidant status and these parameters are considered to be the most commonly used important parameters. DU-145 cells when compared to control group cells exposed to increased boric acid concentrations compared to control caused a significant increase in TOS levels, while concentration-dependent boric acid led to a significant reduction in TAS levels. In conclusion, they found that boric acid inhibited proliferation in this study. They concluded that boric acid treatment triggers oxidative stress by reducing TAS in the DU-145 prostate cancer cell line and increasing TOS levels.[29] On the other hand, in this study on A549 lung cancer cell line, TAS and TOS values were examined but no significant difference was found between them. Poly (ADP-Ribose) Polymerases (PARPs) belong to the family of enzymes that perform multiple cellular processes in addition to DNA repair. PARP1 is best characterized and is one of the PARPs activated by two DNA damage. Poly (ADPRibose) Polymerase (PARP, MA: 116 kDa) is one of the most abundant proteins in the nucleus. It catalyzes the polymerization of ADP-ribose from NAD + molecules in target cellular proteins by attaching to linear or branched polymers. PARP has many roles in most molecules and cellular processes such as DNA damage detection and repair, chromatin modifications, transcription and cellular death pathways. These processes are very critical in physiological and pathological outcomes such as genome repair, carcinogenesis, aging, inflammation, and neuron function.[30] PARP, one of the first identified substrates of caspase has vital role especially in apoptosis and necrosis. In the apoptosis process, caspase 7 and caspase 3 cleave PARP between Asp214 and Gly215 and break down p85 and p25. The PARP cleavage separates DBD from the catalytic domain and inactivates the enzymes. This eliminates PARP activation in response to DNA fragmentation during apoptosis and hinders the necessary ATP consumption in necrotic cell death and the futile efforts required for DNA repair. Thus, PARP cleavage helps the cell enter the apoptotic pathway and is recognized as the distinctive feature of apoptosis.[30,31] PARP works according to the amount of damage in DNA. If DNA damage is very high, PARP leads the cell to necrosis with ATP / NAD consumption. If DNA damage is minimal, PARP, along with other DNA repair enzymes, can help the cell survive. If the caspase is activated, caspase, which plays a critical role in apoptosis from the caspase family and is the main responsible for the cleavage of PARP, goes into cell apoptosis by performing PARP cleavage.[32] The decrease of PARP protein on BPH-1 (Bening Prostate Hyperplasia Cell Line) cell lines to which DPB (Disodium Pentaboratdekahydrate) was applied for 48 hours shows that the DNA doesn't enter into the path of repair mechanism, while an increade in this cleaved

PARP as opposed to PARP protein level indicates that cells enter into the path of apoptosis.[33] In this study, PARP level was higher than the control group and a significant difference was observed between them. No significant difference was observed between the cisplatin group. PARP overactivation leads to more NAD^+ and ATP consumption, which in turn leads to cell dysfunction or necrosis.[34] As a result of the experiment PARP values were higher in the cisplatin group and in the groups where boric acid concentrations were applied. This is due to the fact that PARP leads the cells to necrosis bu incresing NAD and ATP consumption owing to the overactivation of PARP in cisplatin the same reason may be valid for boric acid concentration. In addition, TAS-TOS values were also evaluated. But there was no significant relationship between them. Cancer cells grow and grow uncontrolled. These cells need new vascular formation to grow further. Cancer cells secrete a number of angiogenic factors (VEGF, epidermal growth factor (EGF), interleukin-18). Many of these factors act on different small vessels and bind to receptors in the endothelial cell, resulting in new vessels.[35,28] Based on this information, VEGF values increase in cancer cells and decrease in cytotoxic drugs. In a study, the effect of borax on the treatment process of boron derivatives in human testicular germ cell tumors was investigated and found to be able to produce an anti-invasive effect by suppressing VEGF expression at high doses.[36] In this study, it was observed that boric acid decreased VEGF level compared to control and even cisplatin group.

CONCLUSİON

We can say that various BA concentrations have antiproliferative effect on A-549 lung cancer cell line. We think that the increase in PARP levels leads to cell necrotic death and we can interpret the decrease of VEGF levels as preventing angiogenesis.

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