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NEÜ YAYINEVİ

Boric Acid Shows ER Stress and Apoptosis Mediated Anticancer Activity in Human Pancreatic Cancer MIA PaCa-2 and PANC-1 Cells

Borik Asit İnsan Pankreas Kanseri MIA PaCa-2 ve PANC-1 Hücrelerinde ER Stresi ve Apoptoz Aracılı Antikanser Aktivite Gösterir

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Öz

Amaç: Bu çalışmada borik asitin insan pankreas kanseri MIA PaCa-2 ve PANC-1 hücrelerinde endoplazmik retikulum (ER) stresi ve apoptoz aracılı antikanser etkisinin araştırılması amaçlanmıştır.
Gereçler ve Yöntem: Borik asitin pankreas kanseri hücrelerinin canlılığı üzerine etkisi ve IC_{50} değeri XTT testi ve CompuSyn version 1.0 yazılımı kullanılarak hesaplanmıştır. Apoptotik, anti-apoptotik ve ER stresi ile ilişkili genlerin ifadesi belirlenmiştir. Borik asitin bu hücrelerin koloni oluşum kapasitesi üzerine etkisi ise koloni oluşum testi ile değerlendirilmiştir.
Bulgular: Borik asit zaman ve doz bağımlı olarak her iki hücre hattında da hücre canlılığını baskılamıştır. XTT testi sonucunda MIA PaCa-2 ve PANC-1 hücrelerinde borik asitin IC_{50} dozlarının sırasıyla 15707,5 ve 14248,8 µM olduğu bulunmuştur. Borik asitin her iki hücre hattında da apoptoz ile ilişkili genlerden *BAX, CASP3, CASP8, CYCS* ve *FAS* genlerinin ifadelerini anlamlı derecede arttırdığı gözlenmiştir. PANC-1 hücrelerinde *CASP9* ve *FADD* genlerinin ifadeleride anlamlı derecede yükselmiştir. Borik asitin her iki hücre hattında da ER stresi ile ilişkili *ATF4, HSP47* ve *XBP1* genlerinin ifadesini istatistiksel olarak anlamlı derecede arttırdığı görülmüştür. Ayrıca borik asit muamelesi sadece PANC-1 hücrelerinde *ATF6, CHOP* ve *EIF2A* ifadelerini anlamlı derecede arttırmıştır. MIA PaCa-2 hücrelerinde ise borik asit *GRP78* gen ifadesinin istatistiksel olarak artmasına neden olmuştur. Koloni oluşum testi sonuçları borik asitin her iki hücre hattında da koloni oluşum kapasitelerinin anlamlı derecede baskılandığını göstermiştir.
Sonuç: Borik asit her iki insan pankreas kanseri hücrelerinde hücre canlılığı ve koloni oluşumunu azaltmış olup apoptoz ve ER stresi ile ilişkili genlerin ifadesini değiştirmiştir. Bu bulgular, borik asitin insan pankreas kanseri hücrelerinde ER stresi ve apoptoz aracılı antikanser etkisini göstermektedir.

Anahtar Kelimeler: Pankreas kanseri, endoplazmik retikulum stresi, borik asit, apoptoz

Abstract

Aim: Objective of this study was to investigate the endoplasmic reticulum (ER) stress and apoptosis mediated anticancer effect of boric acid in human pancreatic cancer MIA PaCa-2 and PANC-1 cells.
Materials and Methods: The effect of boric acid on the viability of pancreatic cancer cells and the IC_{50} value were calculated by XTT test and using CompuSyn version 1.0 software. Apoptotic, anti-apoptotic and ER stress-related gene levels were determined. The effect of boric acid on the colony formation capacity of these cells was evaluated with the colony formation assay.
Results: Boric acid inhibited cell viability in these cell lines as time and dose dependent. As a result of the XTT test, the IC_{50} doses of boric acid in MIA PaCa-2 and PANC-1 cells were found to be 15707.5 and 14248.8 µM, respectively. Boric acid significantly upregulated *BAX, CASP3, CASP8, CYCS* and *FAS* expression, which are the genes associated with apoptosis in both cell lines. *CASP9* and *FADD* gene levels were significantly elevated only in PANC-1 cells. It was observed that boric acid statistically upregulated the expression of *ATF4, HSP47* and *XBP1* genes associated with ER stress in both cell lines. In addition, boric acid treatment significantly increased *ATF6, CHOP* and *EIF2A* expressions only in PANC-1 cells. Boric acid also caused an increase in *GRP78* gene expression in MIA PaCa-2 cells. Colony formation test results illustrated that boric acid significantly suppressed colony formation capacities in both cell lines.
Conclusion: Boric acid reduced cell viability and colony formation in both human pancreatic cancer cells and changed gene levels in apoptosis and ER stress pathways. Findings suggested that boric acid exhibits anticancer activity in human pancreatic cancer cells via ER stress and apoptosis.

Key words: Pancreatic cancer, endoplasmic reticulum stress, boric acid, apoptosis

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INTRODUCTION

Pancreatic cancer is the seventh in cancer-related deaths in both genders. Because of poor prognosis in pancreatic cancer, cases and deaths rates are very close to each other. It is estimated that pancreatic cancer can be the third cause of cancer-related deaths by 2025 in a study conducted in 28 European countries (1). Total five-year survival rate of pancreatic cancer is 11% but it is 42% in patients diagnosed with local disease. Because pancreatic cancer is usually diagnosed after the tumor has spread, only less than 20% of patients are eligible for surgery (2). Studies continue for the research of new agents for pancreatic cancer treatment and the development of new treatment strategies.

The endoplasmic reticulum (ER) has critical properties in cellular processes. ER homeostasis can be disturbed due to stress factors such as hypoxia, oxidative insult, hypoglycemia, calcium and ATP depletion. These stress factors affect the correct folding of proteins and can eventually lead to misfolding or accretion of unfolded proteins, resulting in ER stress (3). In the early phase of ER stress, the cell initiates the UPR (unfolded protein response) in response to survive and restore ER homeostasis. PERK (protein kinase RNA-like ER kinase), IRE1 (inositol requiring enzyme1) and ATF6 (activating transcription factor-6) ER transmembrane sensors detect stress and initiate three different UPRs (4). If the time for resolve the unfolded protein event is prolonged, the UPR can stimulate apoptosis pathways via ATF6, PERK and IRE1 signaling pathways (5). Previous literature suggested that ER stress has critical role in antiproliferative properties of many natural compounds having anticancer activity (6, 7). Therefore, inducing ER stress in cancer cells seems to be a potential therapeutic strategy.

Boron is a natural element (8) and Türkiye has approximately 73% of the world's boron reserves and ranks first in the total boron reserves (9). Boron is the ninth most abundant (414 μM) element in seawater (10). Natural boron compounds are used in pharmaceutical formulations due to their antiviral, antibacterial and anticancer effects (11). The anticancer effects of some of the boron derivatives have been demonstrated (12). Mahabir et al. (13) reported that boron intake decreased the incidence of lung cancer in women. Boron can be completely absorbed in the body and passes into all tissues as boric acid (14). Boric acid is a natural component of drinking water and dietary plant products (10). Various studies have shown that boric acid has antibacterial (15), antioxidative (16), anti-inflammatory (17), anticarcinogenic (18), antimutagenic (19), antiinvasive and antiangiogenic (20) properties. Barranco et al. (21) showed that increased boron concentrations in groundwater reduced incidence and mortality of prostate cancer in state of Texas. Also, boric acid (100 and 500 μM for 8 day) showed anti-proliferative effect and increased sensitivity to ionizing radiation in DU-145 human prostate cancer cells (21). This situation is an important reason for elucidating the effect on pancreatic cancer of boric acid at the molecular level.

The effects of boric acid on pancreatic cancer cells were investigated in the context of ER stress, apoptosis and cell proliferation status in this study. For this purpose, XTT, qRT-PCR and colony formation methods were used. Expression levels of genes encoding key proteins in ER stress (*ATF4, ATF6, CALR, CHOP, EIF2A, GRP78, HSP47, IRE1, PERK* and *XBP1*) and apoptosis (*P53, BCL2, BAX, CASP3, CASP7, CASP8, CASP9, CYCS, FAS* and *FADD*) pathways were evaluated using qRT-PCR analysis.

MATERIALS AND METHODS

Chemicals, cell lines and culture conditions

All experimental procedures were approved by the Ethics Committee of N.E.U. Non-drug and Non-Medical Device Research, (2022/3950). Boric acid was commercially obtained from Etimaden and dissolved in DMEM. XTT kit and PBS was purchased from Biological Industries. The human pancreatic cancer cells MIA PaCa-2 (ATCC®CRM-CRL-1420™) and PANC-1 (ATCC®CRL-1469™) were obtained from ATCC and cultured with DMEM (Gibco), 5% FBS (Capricorn Scientific) and 1% penicillin/streptomycin (Biological Industries). These cells were proliferated at 37°C in an incubator including 5% CO_2 and humidified 95% air. QIAzol (Qiagen), cDNA (Bio-Rad) and EvaGreen Supermix (Solis BioDyne) were purchased.

Cytotoxicity

Cells were treated with 10, 50, 250, 1250, 2500, 5000, 15000 and 25000 μM boric acid for 24, 48 and 72 h. The cytotoxic effect of boric acid in these cells was determined by XTT assay using protocol described elsewhere (22). IC_{50} doses of boric acid in MIA PaCa-2 and PANC-1 cells were calculated using CompuSyn Version 1.0 software.

Apoptosis and ER stress-related genes expressions

Total RNAs were isolated using QIAzol and the

Table 1. The Primer Sequences of Studied Apoptosis, ER Stress and Reference Genes in qRT-PCR Analysis.

Gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	PCR product size (bp)
ATF4	TTCGACCAGTCGGGTTTG	GGAGAACCCATGAGGTTTGA	93
ATF6	GAAGGGATCACCTGCTGTTAC	GTCCATCACCTGACAGTCAATC	152
CALR	CGGCTACGTGAAGCTGTT	ACGTTCTTGCCCTTGTAGTT	144
CHOP	AACGGAAACAGAGTGGTCAG	GGTCAGGCGCTCGATTT	137
EIF2A	GGTTTCTTGGCAGCCATTT	TGCAACTTTAGGCTCCTCAC	100
GRP78	TGGTATTCTTCGAGTGACAGC	GACCATCCTTTCAATTTCTTCAGG	108
HSP47	AGATGCAGAAGAAGGCTGTT	GTTCTTGTCGATGGCCTCA	113
IRE1	GCGCATCACAAAGTGGAAGTA	ACATACAGAGTGGGCGTCA	75
PERK	CAAAGTAGATGACTGCAATTACGC	TCCAGCCACGCATTGAAATA	141
XBP1	CCAGAACATCTTCCCATGGAT	GGGTCCAACTTGTCCAGAAT	89
BAX	GGAGCTGCAGAGGATGATTG	GGCCTTGAGCACCAGTTT	151
BCL2	GTGGATGACTGAGTACCTGAAC	GAGACAGCCAGGAGAAATCAA	125
CASP3	GAGCCATGGTGAAGAAGGAATA	TCAATGCCACAGTCCAGTTC	162
CASP7	CGAAACGGAACAGACAAAGATG	TTAAGAGGATGCAGGCGAAG	169
CASP8	GCCCAAACTTCACAGCATTAG	GTGGTCCATGAGTTGGTAGATT	160
CASP9	CGACCTGACTGCCAAGAAA	CATCCATCTGTGCCGTAGAC	153
CYCS	GGAGAGGATACACTGATGGAGTA	GTCTGCCCTTTCTTCCTTCTT	102
FADD	TGACCGAGCTCAAGTTCCTATG	CCAGGTCGTTCTGCTCCAG	108
FAS	GTGATGAAGGACATGGCTTAGA	GCCCAAACTTCACAGCATTAG	156
P53	GAGATGTTCCGAGAGCTGAATG	TTTATGGCGGGAGGTAGACT	129
ACTB	AGCACGGCATCGTCACCAACT	TGGCTGGGGTGTTGAAGGTCT	179

cDNA synthesize was performed by using a Bio-Rad iScript™ cDNA synthesis kit. Primers sequences of apoptosis and ER stress-related genes designed by an online program (https://eu.idtdna.com/site) and were shown in Table 1. qRT-PCR was conducted using the protocol in my previous study (23). *ACTB* was used as an internal control and normalization of qPCR data.

Colony formation assay

The cells were seeded in six-well plates at 10^3 cells/well density. Completed DMEM is refreshed every 3 days for 10 days until colonies become visible. Resulting colonies were methanol fixed for 10 minutes. After 5% crystal violet staining, number of colonies were determined using a light microscope.

Data analyses

All experimental procedures were triplicated. qRT-PCR data were analyzed according to $2^{(-\Delta Ct)}$ method. The comparisons of the groups were assessed using the independent samples t-test in SPSS 26.0 statistical analysis program. In all experiments, $p<0.05$ was accepted as statistically significant.

RESULTS

Boric acid has anti-proliferative effects on MIA PaCa-2 and PANC-1 cells

Effects of boric acid on proliferation of cells was determined using XTT assay. Boric acid suppressed viability of these pancreatic cancer cell lines (Fig. 1).

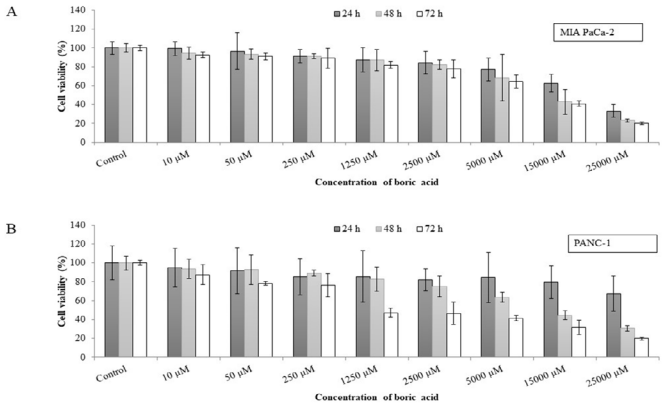

Figure 1. Effects of boric acid on the cell viability in (A) MIA PaCa-2 and (B) PANC-1 cells.

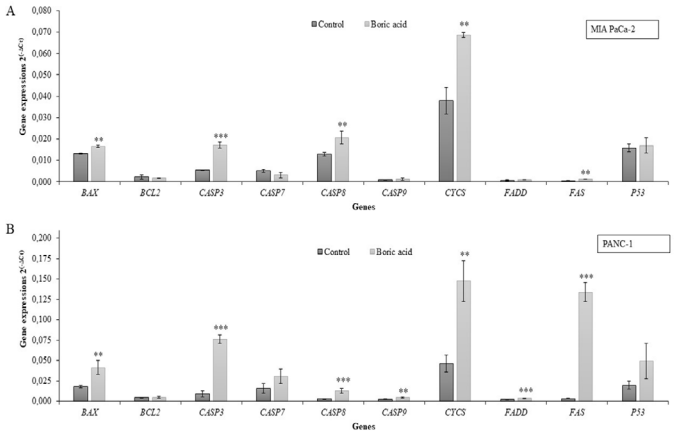

Figure 2. Effects of boric acid on the apoptosis-related genes in (A) MIA PaCa-2 and (B) PANC-1 cells. *P<0.05; **P<0.01; ***P<0.001.

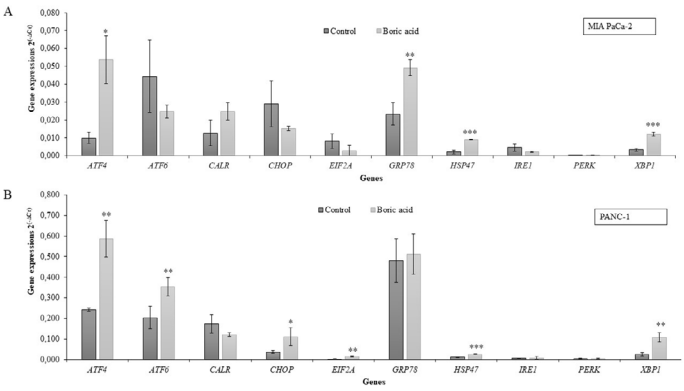

Figure 3. Effects of boric acid on the ER stress-related genes in (A) MIA PaCa-2 and (B) PANC-1 cells. *P<0.05; **P<0.01; ***P<0.001.

The IC_{50} doses of boric acid were 15707.5 µM (MIA PaCa-2) and 14248.8 µM (PANC-1) for 48 h. These doses were used in all subsequent experiments.

Boric acid effects apoptosis-related genes expressions

The effects of boric acid on apoptosis-related genes in pancreatic cancer cells were evaluated using qRT-PCR analysis. It was observed that boric acid caused an upregulated *BAX, CASP3, CASP8, CYCS* and *FAS* gene expressions in MIA PaCa-2 (Fig. 2A, P<0.05). In PANC-1 cell line, boric acid treatment elevated *BAX, CASP3, CASP8, CASP9, CYCS, FADD* and *FAS* gene levels (Fig. 2B, P<0.05).

Boric acid upregulates expression of ER stress-related genes

Expression levels of *ATF4, ATF6, CALR, CHOP, EIF2A, GRP78, HSP47, IRE1, PERK* and *XBP1* that are important in ER stress were evaluated. Boric acid application resulted in *ATF4, HSP47* and *XBP1* upregulation in these cancer cells. Also, a significant upregulation was observed in *ATF6, CHOP* and *EIF2A* gene levels only in PANC-1 cells and in *GRP78* level only in MIA PaCa-2 cells (Fig. 3A, 3B, P<0.05).

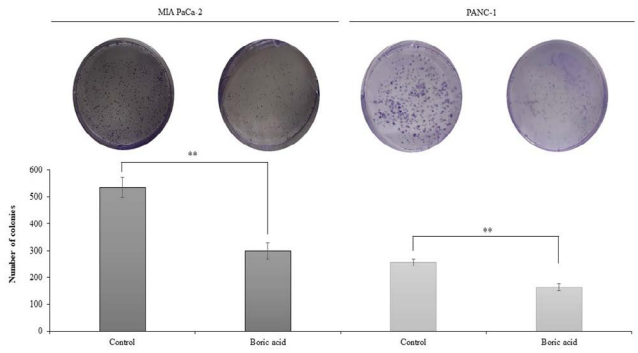

Figure 4. Effects of boric acid on the colony formation in MIA PaCa-2 and PANC-1 cells. **P<0.01.

Boric acid decreases colony formations of MIA PaCa-2 and PANC-1 cells

Effect of boric acid on cells colony formation was determined by using colony assay. The mean number of colonies in MIA PaCa-2 cells were determined as 535±38 in control group and 298±11 in boric acid treated group. On the other hand, mean numbers of colonies in PANC-1 cells were 256±11 in control group and 164±14 in boric acid treated group (Fig. 4, P<0.05).

DISCUSSION

Cancer continues to be a serious health problem for years and the second leading reason of death after heart diseases. Since there are no sufficient treatment options are available, the need for new treatments continues. Scientists are still searching for novel treatments for pancreatic cancer. Scientific interest in boric acid has increased after it was seen that boric acid has anticarcinogenic properties (24, 25). In the present study, the possible anticancer effect of boric acid on human pancreatic cancer cells was investigated by examining cell proliferation, colony formation and expression of genes having critical roles in apoptosis and ER stress pathways.

In a study, researchers reported that boric acid inhibited DU-145 prostate cancer cell proliferation 30% at 100 µM concentration, 60% at 250 µM, and 97% at 1000 µM (26). Hacioglu et al. showed that the IC_{50} and IC_{75} values of boric acid on DU-145 cells were 10.77 and 16.15 mM at 24 hours, respectively. Moreover, boric acid caused cell growth inhibition, apoptosis, decrease in antioxidant levels in dose-dependent manner in these cells (24). In a previous study, IC_{50} value of boric acid was found to be 17 mM in the 48 h in U-87 MG glioblastoma cells (27). It was reported in a study that boric acid decreased the proliferation of DU-145 and LNCaP prostate cancer cells. Eight day 250-1000 µM boric acid treatment inhibited >50% proliferation of DU-145 and LNCaP cells (28). In the present study, the results of the XTT test showed that the IC_{50} doses of boric acid were 15707.5 µM in MIA PaCa-2 cells and 14248.8 µM in PANC-1 cells at 48 h (Fig. 1).

Apoptosis pathway is one of the most important targets in cancer therapy. This pathway must be triggered for the programmed death of cancer cells.

P53, BCL2, BAX, CASP3, CASP7, CASP8, CASP9, CYCS, FAS and *FADD* genes play an important role in this pathway. For this reason, in order to evaluate ER stress-mediated apoptosis, the expressions of *ATF4, ATF6, CALR, CHOP, EIF2A, GRP78, HSP47, IRE1, PERK* and *XBP1* were also investigated. As a result of qRT-PCR analysis, it was observed that *CASP3, CASP8, BAX, FAS* and *CYCS* were upregulated in MIA PaCa-2 cells with boric acid treatment. In addition, boric acid caused increased expression of *CASP3, CASP8, CASP9, BAX, FAS, FADD* and *CYCS* genes in PANC-1 cells (Fig.2). In ER stress-related genes, boric acid increased the levels of *ATF4, HSP47* and *XBP1* genes in both cell lines. Moreover, boric acid caused increased gene expression of *ATF6, CHOP* and *EIF2A* only in PANC-1 cells, and *GRP78* gene expressions only in MIA PaCa-2 cells (Fig. 3).

In a previous study, boric acid upregulated ATF4, ATF6 and eIF2α protein levels in prostate cancer DU-145 cells (29). In a study, it was shown that boric acid induced ER stress in kidneys of rats with cisplatin nephrotoxicity (25). Boric acid has induced ER stress by activating eIF2a, GRP78/BiP, and ATF4 in DU-145 prostate cancer cells in the previous study (30). Studies have shown the effect on ER stress of boric acid in prostate cancer DU-145 cells (29, 31) and in rats with cisplatin nephrotoxicity (25). However, studies investigating boric acid effects on ER stress is still quite limited.

A previous study showed increased arrest in the G2/M phase of the cell cycle in boric acid-treated HepG2 human hepatocellular carcinoma cells. Also, boric acid treatment caused an increase in tumor suppressor P53 but a decrease of anti-apoptotic gene *BCL2* level in HepG2 cells (32). Boric acid increased expression levels of *BAX* and *CASP3* apoptotic genes in DMS-114 small-cell lung cancer cells. Expression levels of *BIRC-2, BIRC-5* and *BCL2* anti-apoptotic genes decreased in boric acid treatment groups (12). It was showed that boric acid inhibited proliferation, migration, invasion and colony formation of ovarian cancer MDAH-2774 cells. It was also reported that boric acid increased expression of *BAX, BID, CASP3* and *CASP9* apoptotic genes, and decreased anti-apoptotic genes *BCL2* and *BCL-XL* (33). In a study conducted on DMS-114 small-cell lung cancer cells, boric acid reduced the colony formation abilities of these cells (12) In the present study, boric acid reduced the colony formation capacity of pancreatic cancer cells (Fig. 4).

In conclusion, boric acid showed anticarcinogenic effect by acting expression of apoptosis and ER stress related genes in human pancreatic cancer cells. Further analyzes and in vivo studies are needed to possible application of boric acid in the treatment of pancreatic cancer.

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